CHEMOTHERAPY AND DRUG TARGETING IN THE TREATMENT OF LEISHMANIASIS

ANNUAL REPORT

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MAY 30, 1990

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7146

University of Massachusetts
Amherst, Massachusetts 01003

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER Three	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)* Chemotherapy and Drug Targeting in the Treatment of Leishmaniasis		5. TYPE OF REPORT & PERIOD COVERED May 15, 1989 - May 14, 1990 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Linda L. Nolan		8. CONTRACT OR GRANT NUMBER(s) DAMD17-87-C-7146
9. PERFORMING ORGANIZATION NAME AND ADDRESS Division of Public Health University of Massachusetts Amherst, MA 01003		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research & Development Command, Fort Detrick, Frederick, MD 21701-5012		12. REPORT DATE May 30, 1990 13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*		15. SECURITY CLASS. *(of this report)* Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Leishmania, mode of action of purine anologs, DNA polymerase, S-adenosylmethionine synthetase, microplate assay for drug screening.

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Leishmaniases, a disease caused by protozoan parasites of the Leishmania spp., is one of the major public health problems currently affecting humanity. Therapeutic agents for this disease is either ineffective or toxic. The purpose of this work is to aid in the development of an effective, non-toxic treatment of leishmaniasis.

The objectives of this research are the following:

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(2) To test promising anologs both in vivo and in vitro to determine their potential as antileishmanial agents.

(3) To develop a more rapid and economical procedure for in vivo screening of potential antileishmanial compounds.

(4) To determine the potential toxicity to human cells of promising antileishmanial compounds.

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ABSTRACT

Leishmaniases, a disease caused by protozoan parasites of the Leishmania spp., is one of the major public health problems currently affecting humanity. Therapeutic agents for this disease is either ineffective or toxic. The purpose of this work is to aid in the development of an effective, non-toxic treatment of leishmaniasis.

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(3) To develop a more rapid and economical procedure for in vivo screening of potential antileishmanial compounds.

(4) To determine the potential toxicity to human T_4 cells of promising antileishmanial compounds.

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II. BACKGROUND

Species of the parasitic protozoan genus Leishmania are the causative agents of a wide variety of human cutaneous, mucocutaneous, and visceral diseases. These organisms reside throughout their digenetic life cycles in different environments. The extracellular, flagellated promastigote forms reside in the alimentary tract of their sandfly vector hosts and the obligate intracellular amastigote forms exists within the phagolysosomal system of macrophages in their mammalian hosts. How these organisms transform, survive, and respond to signals within their infected hosts is unknown.

Most of the major metabolic pathways in the parasitic protozoa have been reported to be similar to those of the mammalian host except for nucleic acid metabolism (1,2,3,4). This pathway is unusual in several ways. First, they lack the ability to synthesize the purines de novo, making them entirely dependent on the salvage pathway for their supply of purine nucleotides. Second, many of the enzymes involved in nucleic acid biosynthesis either have unusual substrate specificities or unusual subcellular localizations (1-39). Third, a large proportion of the DNA which is produced is incorporated into a unique organelle known as the kinetoplast. Kinetoplast DNA the mitochondrial DNA of leishmania and related parasitic protozoa, has a remarkable structure. It consists of networks, of thousands of interlocked DNA circles, and each cell has one network within its single mitochondrion (40-43). Nothing is known either about the function of kinetoplast minicircles or the reason that these molecules are interlocked, together with maxicircles, in an enormous network. Neither is it known why these parasitic protozoa, alone among the eukaryotes, have their mitochondrial DNA organized in this unusual way. Fourth, the major DNA polymerase isolated from the parasitic protozoa has been shown to have different characteristics than its mammalian counterpart and to be immunologically distinct (44-47).

The presence of multiple DNA polymerases in eukaryotic cells is a well established fact. The use of specific inhibitors has helped to characterize nuclear and organelle DNA polymerases. DNA polymerase α involved in the replication of the nuclear genome is strongly inhibited by aphidicolin regardless of the source of the enzyme. Other eukaryotic DNA polymerases like the β-polymerase involved in DNA repair, as well as the chloroplastic and mitochondrial polymerases are not affected by this drug (48-49).

Chang et al. (1980) reported that extracts of bloodstream forms of Trypanosoma brucei showed that both DNA polymerase α and DNA polymerase β activities were present (42). The detection of DNA polymerase α in T. brucei demonstrated the presence of this enzyme in unicellular organisms. Chang also stated that DNA polymerase α was present in L. mexicana. They found the DNA polymerases in T. brucei to be immunologically distinct from host enzymes, and suggested that the structural differences between the parasite and the host enzymes could be exploited for the development of agents to combat parasitic diseases. Dube et al. (49) reported on the detection and characterization of DNA polymerase α in T. brucei and found that specific antisera that cross-reacted with mammalian DNA polymerase from different species failed to cross-react with the trypanosome polymerase (49).

Investigations in our laboratory involving isolation, and characterization of DNA polymerase in Leishmania mexicana have shown it to have many biochemical differences when compared to mammalian DNA polymerase.

Sinefungin

We have been investigating inhibitors of nucleic acid synthesis, and have found sinefungin to be extremely inhibitory. ED_{50} for growth of promastigotes of L. mexicana sp. was as low as 5nM and up to 100uM.

Sinefungi a natural nucleoside isolated from cultures of Streptomyces incarnatus and S. griseolus, is structurally related to S-Adenosylhomocysteine (SAH) and S-Adenosylmethionine (SAM) (Fig. 1). Sinefungin has been shown to inhibit the development of various fungi (50) and viruses (51-53) but its major attraction to date resides in its potent antiparasitic activity (54-62).

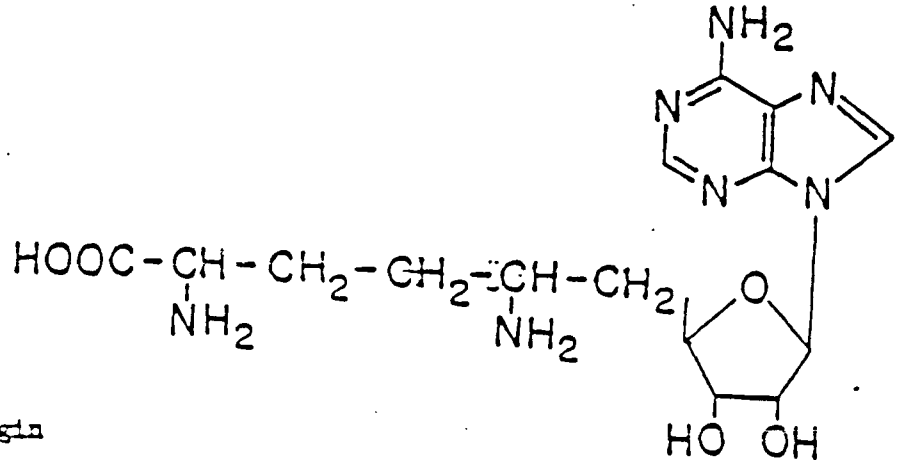
Evidence from our laboratory and reports in the literature have implicated at least eight possible targets for the inhibitory action of sinefungin in various systems (50-62):

1. DNA methylase
2. S-adenosylmethionine decarboxylase
3. S-adenosylmethionine synthetase
4. S-adenosylmethionine hydrolase
5. Protein carboxymethyl transferase
6. mRNA methyltransferase
7. Guanine 7 - methyltransferase
8. Phospholipid methylation - which results in loss of membrane fluidity and receptor function

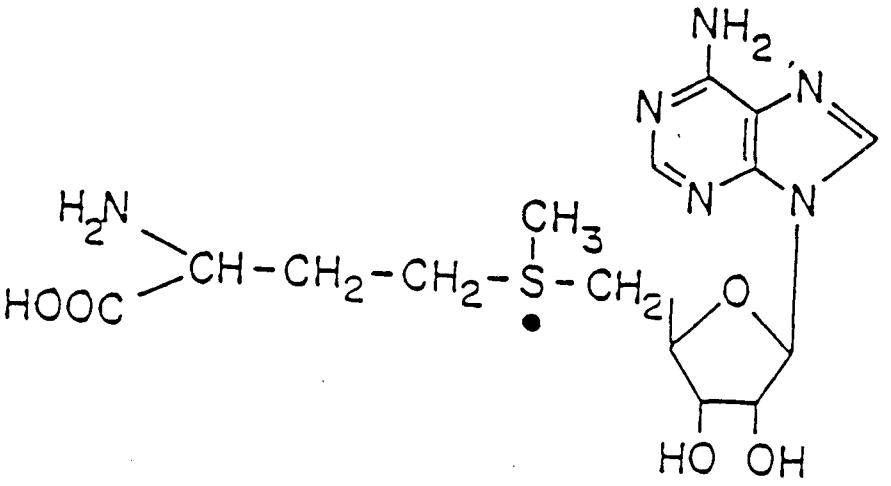
In nearly all biochemical reactions involving the transfer of intact alkyl groups, the nucleoside of amino acids (SAM) (63) and its decarboxylated derivative (DSAM) (64) are the requisite of alkyl donors. Only in the methylation of homocysteine (65) to form methionine (66) is another alkyl donor, 5'-methyltetrahydrofolate or betaine, utilized. The methionine synthesized is converted to SAM as a result of nucleophilic attack by the sulphur atom of methionine on the 5'-methylene carbon of ATP (Fig. 2). SAM is either employed directly as the methyl donor in a wide variety of enzyme-catalyzed methylation reactions or is first decarboxylated to DSAM which serves as a source of aminopropyl groups in the biosynthesis of the polyamines spermidine and spermine (61). The nucleoside products of the enzyme-catalyzed methyl transfer and aminopropyl are S-adenosylhomocysteine (SAH) (62) and 5'-deoxy-5'-methylthioadenosine (MTA) (63). The metabolism of SAH is of great importance in the regulation of cellular transmethylation reactions using SAM as methyl donor, or other reactions in which SAM participates as an allosteric effector (64.65). Changes in the concentrations of SAM and SAH affect the methylation and the properties of such molecules as nucleic acids, proteins, phospholipids and carbohydrates.

Bases modified by methylation have been known to occur at a low frequency in DNA for more than three decades (72.73). This modification of DNA is carried out by specific methyltransferases (DNA methylases) that transfer the chemically active methyl group from S-adenosylmethionine to either carbon 5 of cytosine residues or the exocyclic amino group attached to

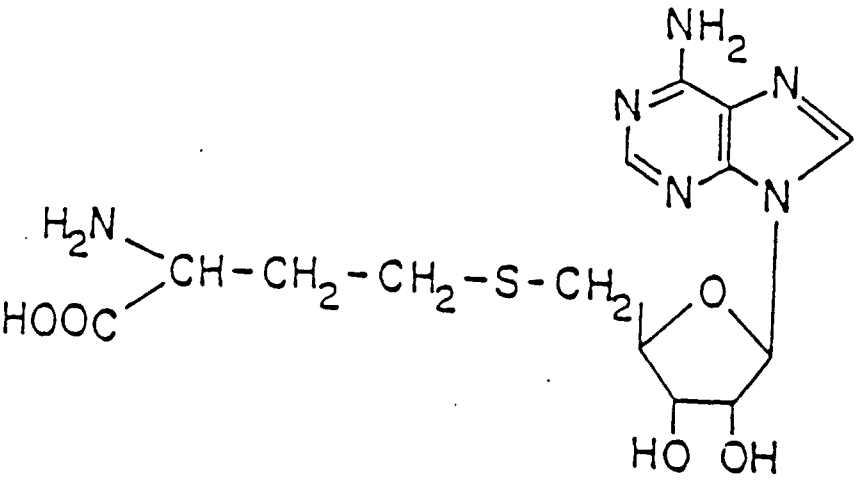
Fig. 1 Structure Sinefungin



Sinefungin



SAM



SAH

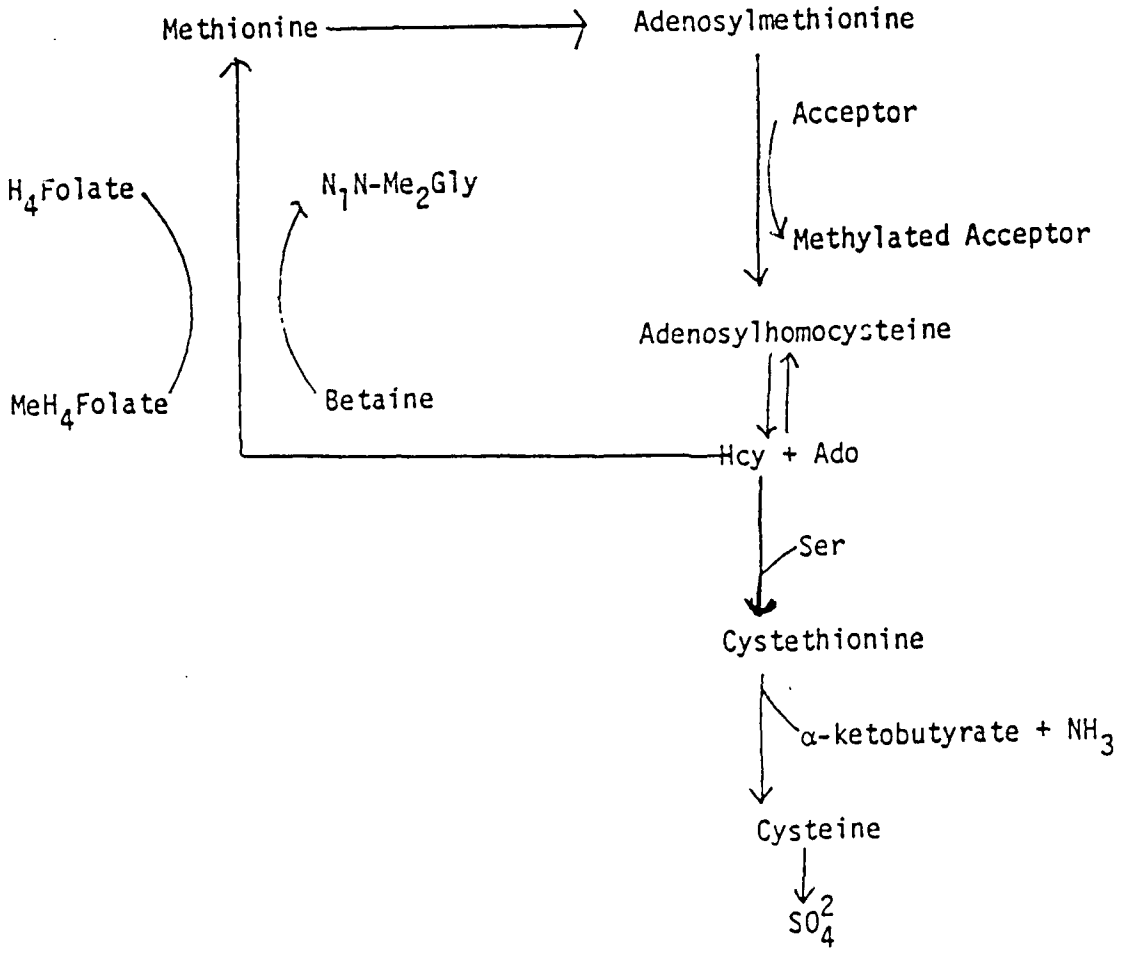


Fig. 2. Pathway for the metabolism of S-adenosylhomocysteine

Hcy = L-homocysteine

Ado = Adenosine

Adapted from: PURINE METABOLISM IN MAN - IV
Edited by Chris H. M. DeRruyn, H. Anne Simmonds, and Mathias Muller
(Plenum Publishing Corporation, 1984)
P. K. Chiang, p. 199

carbon 6 of adenine residues of the DNA chain (74). The widespread occurrence of methylated bases in DNA of various organisms, the sequence specificity of the various DNA methylases and the nonrandom distribution of the methylated bases along the chromosomes strongly suggest that modified bases in DNA are biologically significant.

Modified nucleosides have been found in tRNA and appear on a specific location on the coverleaf structure. In spite of the fact that the physiological role of these modified nucleosides is not fully understood, it appears to confer to the tRNAs some structural characteristics essential for their function. Sinefungin inhibits Rous sarcoma virus (RSV) induced foci formation (53,75-76) and has been found to inhibit tRNA-base methylation in chick embryo fibroblasts infected by RSV (76).

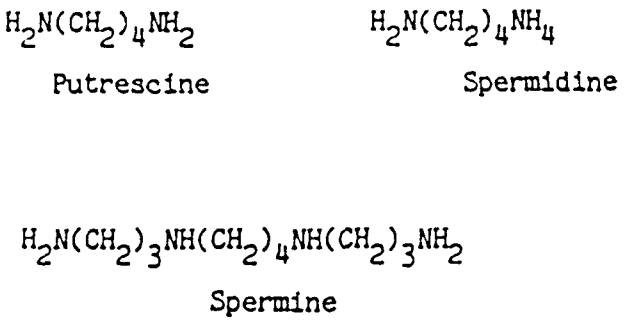
SAM is also a precursor to the polyamines (Fig. 3). The naturally occurring polyamines putrescine, spermidine and spermine are organic cations widely distributed in both procaryotic and eukaryotic organisms (77-79). Polyamine synthesis increases and polyamine levels rise when the growth rate is maximal. Growth appears to be related to and dependent upon polyamine biosynthesis. The polyamines are also found in DNA and RNA viruses of bacteria (80) plants and animals. The putrescine, spermidine and Mg^{2+} present in bacteriophage T_4 are sufficient to neutralize a large part of the phage DNA (81). Turnip yellow mosaic virus (TYMV) and some other plant viruses have been shown to contain spermidine (80). Spermidine has been found in the RNA isolated from R17 phage (83) and from TYMV (84). Infection of E. coli by R17 leads to a marked accumulation of spermidine, which parallels that of RNA (85).

In 1948, Herbst and Snell (86) identified putrescine as a factor essential for the growth of Hemophilus parainfluenzae. Since that time, polyamines have been shown to be stimulatory to (or essential for) the growth of various organisms, from bacteria (87-90) and fungi (91) and cultured animal cells (92).

Polyamines have a stimulatory effect on normal RNA synthesis. Uridine incorporation is stimulated by the addition of polyamines to various organisms (87,88) and organelles (95). In in vitro systems, polyamines increase RNA synthesis with RNA polymeraseses isolated from several organisms (96-98), both by enhancing the binding of polymerase to DNA templates and by displacing RNA products. Spermidine has differential effects on the selection of cistrons to be transcribed in some phage systems (99). Polyamines are also important in steps of DNA replication, as in the marked stimulation of T_4 polynucleotide kinase (93) and in the activity of various DNA polymerases (101-103).

As regards protein synthesis, the polyamines help to maintain ribosomal structure (104,105) as well as to facilitate the assembly of ribosomal subunits (106). Spermidine appears to be required for the function of ribosomes isolated from one organism (107) and is a component of the ribosomal association factor in another (108). Polyamines can activate the formation of aminoacyl-tRNA in the absence of Mg^{2+} (109-111). Spermidine and spermine can stimulate the in vitro incorporation of amino acids into protein in numerous systems (103,113,115), and can replace substantial portions of the Mg^{2+} requirements in such systems; e.g., by various combinations plus suboptimal concentrations of Mg^{2+}, either spermidine or

Figure 3



Structures of the common polyamines

putrescine plus spermidine increase the poly(u)-directed or R17-RNA-directed synthesis of protein to a level attained in the presence of optimal Mg^{2+} concentrations alone (83).

Because of their cationic nature, polyamines bind strongly to nucleic acids. Tsuboi (116) and Liquori et al. (117) postulated a model for the interaction of polyamines with double-helical nucleic acids to explain the stabilization of nucleic acids by polyamines (118-119).

In conclusion, the antifungal agent, sinefungin, is a structural analogue of S-adenosylmethionine. In various systems, sinefungin has been shown to be an inhibitor of transmethylation reactions in the production of creatine (120), sterols (121), phospholipids (122), cyclopropane fatty acids (123), and methylated proteins (76,124). For RNA molecules, sinefungin has been reported to inhibit methylation of tRNA (76) and production of the "cap" structure at the 5' terminus of eukaryotic mRNA (51). Recently, it has been reported that sinefungin slows methylation of rRNA, and is associated with differential loss of undermethylated 18 S rRNA species (125).

Our laboratory has been investigating the inhibitory action of sinefungin in the parasitic protozoa Leishmania. We studies macromolecular synthesis and found that only DNA synthesis was drastically inhibited in these organisms in the presence of sinefungin and that this inhibition was not the consequence of either nucleoside uptake or its phosphorylation or direct inhibition of DNA polymerase.

Our work this past year has focused on the isolation and characterization of S-adenosylmethionine synthetase, an enzyme responsible for the methylation of various proteins, and nucleic acids. This enzyme is known to be inhibited by compounds similar to sinefungin.

Isolation of L.mexicana DNA Polymerase:

A 12.7g L.mex cell pellet was resuspended in 20mL of buffer A and sonicated twice for 45 seconds at maximum output of a Braunsonic 2000 sonicator, centrifuged 90 min at 45,000xg, 2°C. The supernatant (17mL) was filtered through a layer of glass wool and loaded on top of a 56.5mL (11.5 x 2.5cm) DEAE (DE23, Whatman) column equilibrated in buffer B at a flow rate of .5mL/min. Absorbance was monitored at 280nm and the first peak detected containing unbound proteins was collected as a batch (46mL), and assayed for DNA Polymerase α and ß activity (Fraction I). Fraction I was dialyzed overnight against 2L of buffer C, changed to 2L of fresh buffer C for 2 hours and loaded on top of a 10.6mL (6 x 1.5cm, 10.6mL) cellulose phosphate (P11, Whatman) column equilibrated with buffer C at a flow rate of .5mL/min. The sample was washed into the column with 5 column volumes of buffer C, followed by 3 column volumes of .15M KCl in the same buffer. DNA Polymerase was eluted from the column with four column volumes of a .4M KCl step in buffer C. Assay of the column eluant confirmed enzyme activity in the .4M KCl batch to give fraction II. Fraction II was precipitated with 70% (NH4)2SO4 and centrifuged 10 minutes at 30,000xg and stored at -70°C until used again. The pellet was resuspended in 2.5mL of buffer D, and loaded on a (1.5 x 88cm, 156mL) Sephacryl S-300 gel filtration column equilibrated in buffer D at a flow rate of .2mL/min. The sample was washed with buffer D and fractions were collected every 10 minutes (2mL). Every other tube after the void volume (Vo = 44mL, determined with Blue Dextran 2000) was assayed for DNA Polymerase α and ß activity. Active fractions (Fraction III enzymes) were stored at -70°C.

Enzyme Assays:

Enzyme activity was assayed in a final volume of 50uL for 30 minutes at 35°C. The assay mix consists of:

50mM Tris, pH 8.0*
50mM Ammediol, pH 9.0#
50uM each of dATP, dCTP, dGTP
1mM DTT
8mM MgCl2*/15mM MgCl2#
400ug/mL Activated Calf Thymus DNA
10uL of enzyme sample
3HTTP at 400-450 CPM/pmole

*(α assay only)
#(ß assay only)
BSA (100ug/mL) was included in the assay mix to test Fraction II and Fraction III enzymes.

Drug inhibition study, Effect of Antibody:

To test for N-ethylmaleimide inhibition the enzyme was preincubated 10 minutes at 35°C with the drug at 1, 5, or 10mM. Inhibition by dideoxythymidine tri-phosphate (ddTTP) at 10, 25, 50, 75, 100, 500, and 1000uM was studied by including the inhibitor at the specific concentration during the assay.

The enzyme was pre-incubated with a monoclonal antibody against Human DNA Polymerase α (30ug/mL) at 35°C for 10 minutes and then assayed under normal assay conditions.

Buffers:

A: Sonication Buffer
.25M Potassium Phosphate, pH 7.4
1mM Benzamidine HCl
1mM EDTA
1mM DTT
1% DMSO
20% Glycerol

B: DEAE Buffer
.20M Potassium Phosphate, pH 7.4
1mM Benzamidine HCl
1mM EDTA
1mM DTT
1% DMSO
20% Glycerol

C: P11 Buffer
50mM Potassium Phosphate, pH 7.4
1mM Benzamidine HCl
1mM EDTA
1mM DTT
1% DMSO
20% Glycerol

D: Sephacryl S-300 Buffer
50mM HEPES, pH 7.5
1mM DTT
.5M NaCl
20% Glycerol

Density Gradients

A 5 to 20% sucrose solution density gradient was prepared in 25 mM Potassium phosphate buffer, pH 7.4, 1 mM dithiotreitol (DTT), and .15 M KCl. Centrifugation was for 16 hrs at 4°C at 40,000 rpm in a Beckman sw 41 Ti rotor. Gradients were fractionated by displacement from the bottom with 50% sucrose solution containing 2 mM potassium hydrogen phthalate at flow rate of .75 mL/min. Fractions were collected every 30 seconds (.375 mL) and assayed for DNA polymerase α and ß activity (no N-ethylmaleimide present in the ß polymerase assays).

Activity gels:

In order to identify a protein band with DNA polymerase activity and identify its molecular weight, the active fractions obtained from Sephacryl S-300 HR gel filtration chromatography (fraction IV) were electrophoresed under native (non-denaturing) conditions. One µg of enzyme fraction per lane was loaded on a 4-30% polyacrylamide gradient gel (8 x 7 cm). The gel was run at 4°C at 70 V constant voltage for 20 minutes until all of the sample entered the gel. The voltage was increased to 150 and the gel was run for 18 hrs. Lanes containing the enzyme fractions run side by side were sliced and one lane was used for silver staining and the other was sliced in 2 mm sections from top to bottom and each section was placed in a micro-centrifuge tube. To each slice was added 3 slice volumes of DNA polymerase assay mix and incubated overnight at 4°C. The slices were then incubated 1 hour at 35°C and processed for acid insoluble counts.

Synthetic Template Assays:

The ability of the DNA polymerase α to utilize synthetic polydeoxynucleotides as templates was tested with the standard assay mix. Activated DNA was replaced by Poly dA:Oligo dT or by poly dC:Oligo dG in the assay mix. For the poly dC:Oligo dG assays, ^{3}H-dGTP (350 CPM/pmole) was the labeled nucleotide. In these assays the radioactive nucleotide substituted all four dNTP's.

Both DNA polymerase α and ß were tested in their ability to use synthetic polyribonucleotides as templates. The α polymerase was assayed in the presence of 20 mM MES buffer, pH 6.9, 1 mM DTT, .5 mM $MnCl_2$, and 100 µg/mL denatured BSA, and .08 units of synthetic polyribonucleotide template. The ß polymerase was assayed in the presence of 20 mM Ammediol buffer, pH 9.0, .5 mM $MnCl_2$, 100 µg/mL denatured BSA, and .08 units of synthetic polyribonucleotide template.

Affinity Chromatography:

An additional purification step for DNA polymerase α was attempted by using DNA cellulose affinity chromatography. The α polymerase fraction IV was extensively dialyzed against DCC buffer (50 mM Tris pH 7.5, 20% glycerol, 1 mM DTT, 1 mM EDTA, 50 mM KCl) and loaded on a 1.5 x 2.3 cm (8 mL) DNA cellulose column at a flow rate of .17 mL/min. The column was washed with 8 column volumes of DCC buffer and proteins eluting out of the column were collected as a batch. The column was then washed with 4 column volumes of DCC buffer containing .2 N KCl and the eluting peak of A_{280} was collected as a batch (10 mL). The column was washed further with 4 column volumes of .6 N KCl in DCC buffer and the eluted proteins were collected as a batch. The fractions were assayed for DNA Polymerase α and ß activity.

Precipitation of nucleic acid:

In an effort to shorten up the isolation protocol and to try to increase the number of counts obtained from fraction II (DEAE Chromatography) we

have used protamine sulfate to precipitate the endogenous nucleic acid. The crude extract (fraction I) was added a 1/10 volume of a 2% protamine sulfate solution prepared fresh in buffer A (200 mM KPO_4 pH 7.4, 1 mM DTT, 1 mM EDTA, 1 mM Benzamidine, 1% DMSO). The extract was stirred for 30 minutes at 4°C and centrifuged 15 min at 27,000 x g, 4°C. The supernatant was used to continue with the isolation and the pellet was discarded.

Inhibition by pyrophosphate analogues:

L. mexicana DNA polymerase α was tested for inhibition by several known mammalian DNA polymerase inhibitors that are analogues of pyrophosphate (PP_i). Each inhibitor was preincubated in ice for 30 minutes with the enzyme prior to incubation at 35°C for 30 minutes and assayed in 65µL of a mix containing 20 mM MES (pH 6.9), 50 µM each of dATP, dCTP, dGTP, 1 mM DTT, 8 mM $MgCl_2$, and 40 nM ^{3}H-TTP (375 CPM/pmole).

RESULTS AND DISCUSSION

The sedimentation coefficient for the DNA polymerase α was determined to be 6.8 S. For the DNA polymerase ß the value was determined at 3.4 S (Figure 5). When a standard curve of log MW vs. log S is constructed using ferritin, alcohol dehydrogenase, BSA, and cytochrome c as the standards, a molecular weight of 130,000 is found for DNA polymerase α and a MW of 45,000 is found for DNA polymerase ß. Previous attempts to determine the sedimentation constant of these two enzymes were hampered by the low counts obtained. Lowering the ionic strength of the buffer where the gradients were made resulted in an increase in enzyme activity that allowed the identification of the fractions containing the enzyme. Earlier attempts at density gradients had resulted in very low enzyme activity.

Due to the very little enzyme isolated from cells it has not been possible to obtain enough material to load in polyacrylamide gels and to recover any enzyme activity from the gels. We are in the process of scaling up the isolation protocol in order to obtain larger quantities of the enzymes to allow us to reattempt to recover enzyme activity from polyacrylamide gels.

Assay of the DNA polymerases with synthetic templates showed that both α and ß polymerases can use poly A:$(dT)_{10}$ as template almost as efficiently as activated DNA (Table 1). The labeled nucleotide used for the assay will directly influence enzyme activity. DNA polymerase α will use ^{3}H-TTP 9.5 times more efficiently than ^{3}H-dGTP when activated DNA is the template, DNA polymerase ß is 4.3 times more efficient with ^{3}H-TTP than with ^{3}H-dGTP. This difference could be due to the template source since the activity of the DNA polymerase α is about the same with both labelled nucleotides when poly dA:oligo dT or poly dC:oligo dG are used as templates. We are currently testing DNA polymerase ß for its ability to use the same synthetic templates.

Preliminary results indicate that affinity chromatography with DNA cellulose could improve the specific activity of the enzymes. However, we again face the problem of small amounts of starting material and the yield from the column is too small. We are currently isolating more enzyme and accumulating material in order to look at the DNA cellulose column fractions using gel electrophoresis and determine their composition.

Removal of endogenous nucleic acid from the crude extract with protamine sulfate has the advantage of being a fast and reliable method; the nucleic acid is precipitated and no enzyme activity is found in the pellet. No loss of activity is detected when compared to the alternate method of removing nucleic acid (DEAE chromatography).

Assay of DNA polymerase α in the presence of pyrophosphate analogues results in a selective inhibition by certain analogues that seems to be different from the inhibition of DNA polymerase α from calf thymus by the same compounds. Table 2 shows that FPAA inhibits L. mexicana DNA polymerase α with an IC_{50} of 150 μM whereas it inhibits Calf thymus DNA polymerase α with an IC_{50} of 20 μM (Talanian, et al.(1989) Biochemistry 28, 8270). On the other hand, F_2PAA was inactive against Calf thymus DNA polymerase α, but it inhibited L. mexicana DNA polymerase α with an IC_{50} of 850 μM (Table 2).

Table 1. Template activity for the L. mexicana DNA polymerases.

	pmoles of labelled nucleotide/30 min	
TEMPLATE	α	ß
With ^{3}H-TTP:		
Activated DNA	9.5	4.3
poly A:$(dT)_{10}$	8.8	4.8
poly dA:oligo dT	30.6	Not Tested
With ^{3}H-dGTP:		
Activated DNA	1.0	1.0
poly C:$(dG)_{12-18}$	1.8	0.3
poly dC:oligo dG	26.5	Not Tested

Table 2.Inhibition of DNA Polymerase α by pyrophosphate analogues:

Compound	IC_{50} (μM) L. mexicana	IC50 (μM)* Calf Thymus
BrPAA	I*	160
ClPAA	I	120
FPAA	150	20
COMDP	350	300
COPAA	600	170
F_2PAA	850	I

*I = Compound did not inhibit the enzyme at the concentration range tested.
*From Talanian et al. (1989) Biochemistry 28, 8270.

Fig. (4-13) and Tables (1-11) summarize some of the characteristics and toxicity studies we have during the last year.

SEPARATION OF DNA POLYMERASES

Figure 4

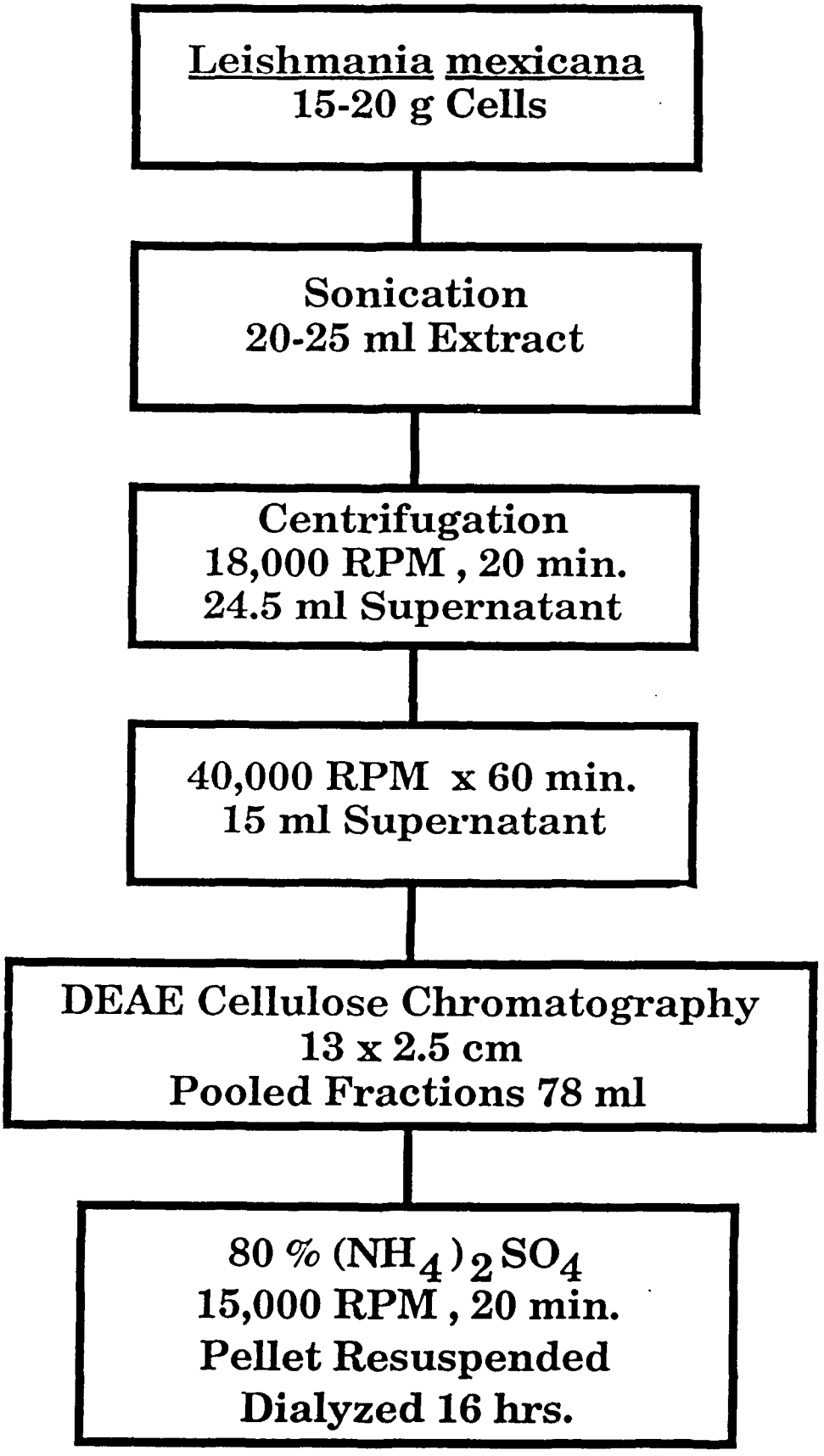


Figure 4

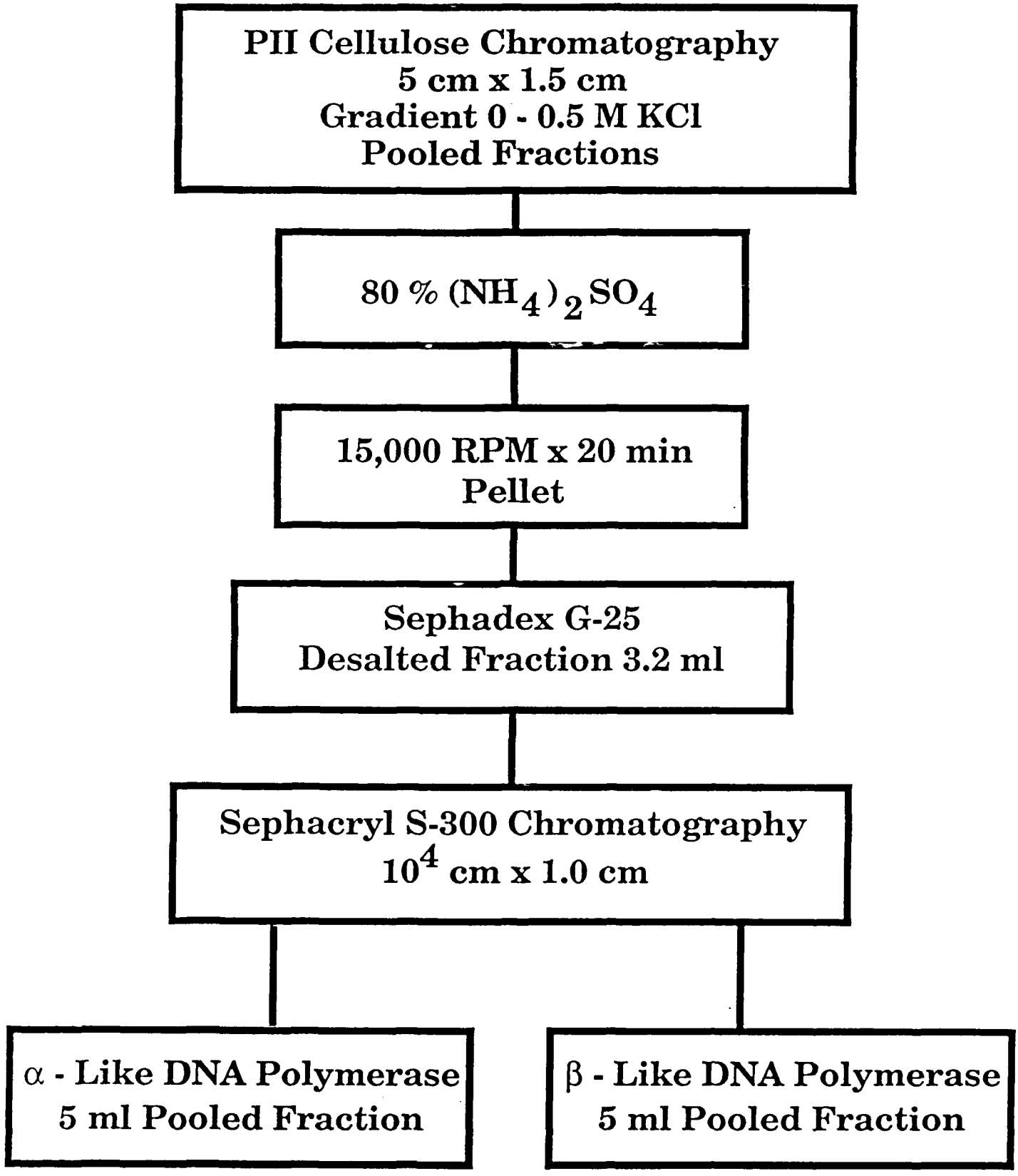

PII Cellulose Chromatography
5 cm x 1.5 cm
Gradient 0 - 0.5 M KCl
Pooled Fractions
80 % $(NH_4)_2SO_4$
15,000 RPM x 20 min
Pellet
Sephadex G-25
Desalted Fraction 3.2 ml
Sephacryl S-300 Chromatography
10^4 cm x 1.0 cm
α - Like DNA Polymerase
5 ml Pooled Fraction
β - Like DNA Polymerase
5 ml Pooled Fraction

Figure 5

Sedimentation of L. Mexicana DNA Polymerases in a 5-20% Sucrose Density Gradient

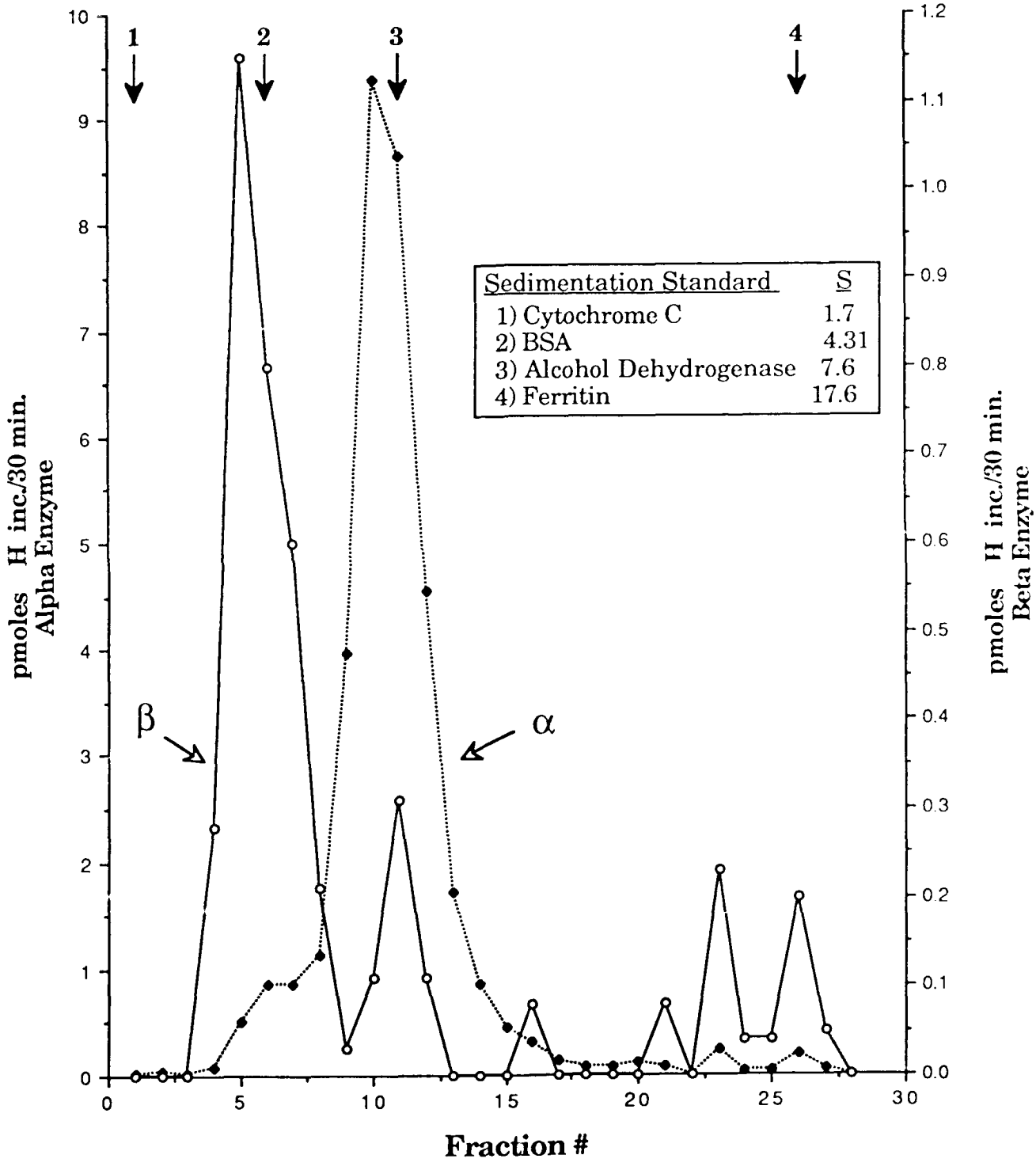


Figure 6

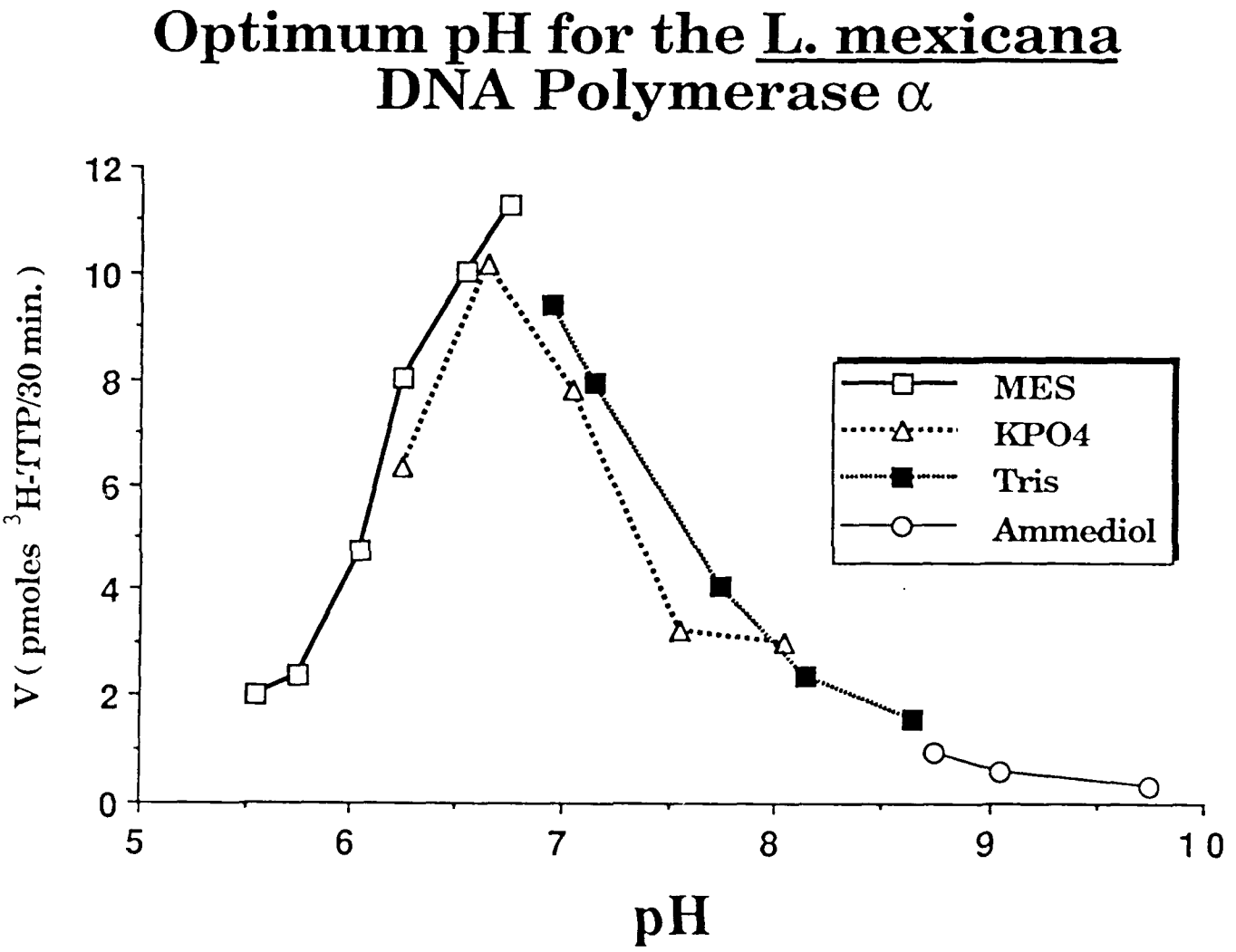

Optimum pH for the L. mexicana DNA Polymerase α
V (pmoles 3H-TTP/30 min.)
12
10
8
6
4
2
0
5
6
7
8
9
10
pH
MES
KPO4
Tris
Ammediol

Figure 7

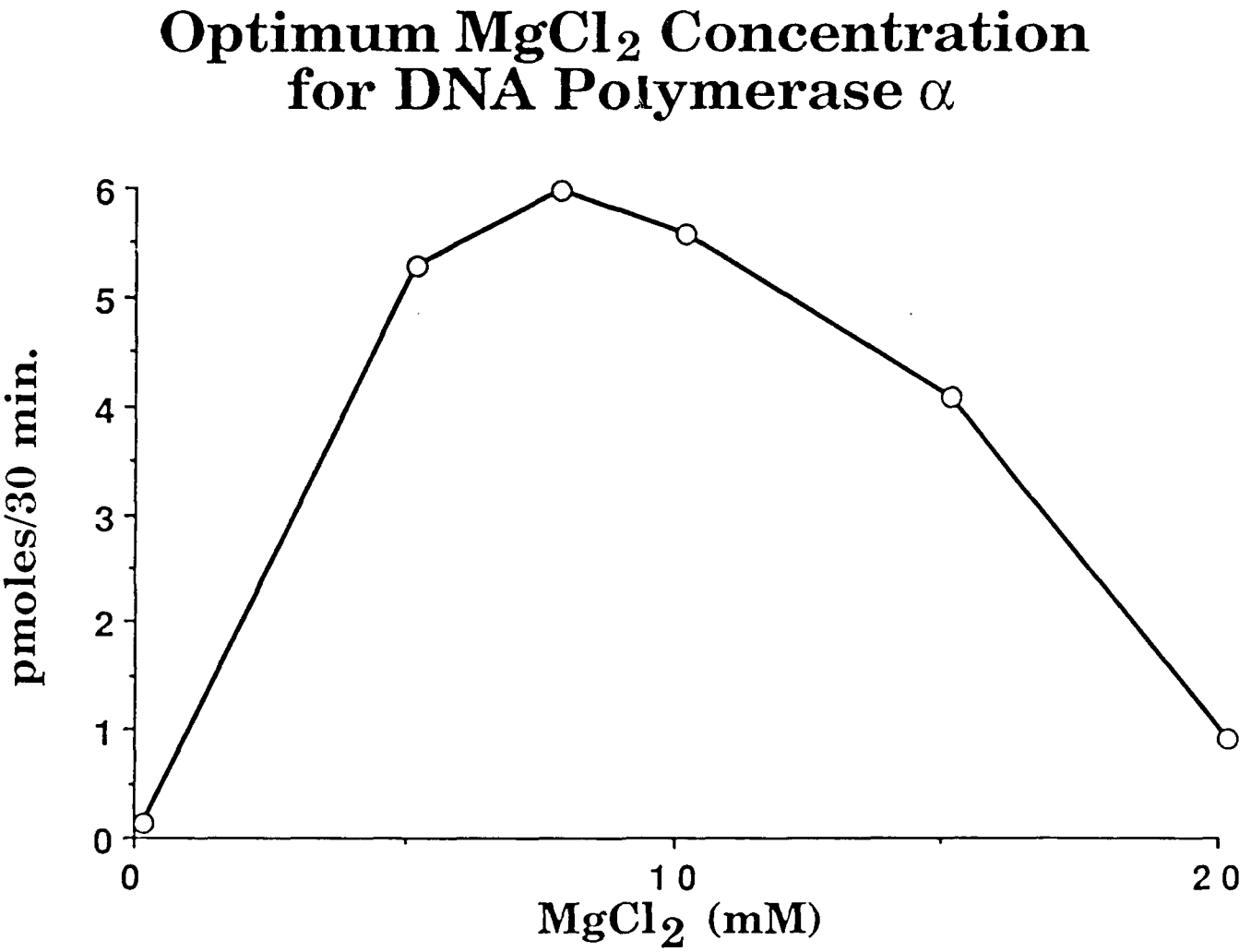

Optimum $MgCl_2$ Concentration for DNA Polymerase α
pmoles/30 min.
0
1
2
3
4
5
6
0
10
20
$MgCl_2$ (mM)

Figure 8

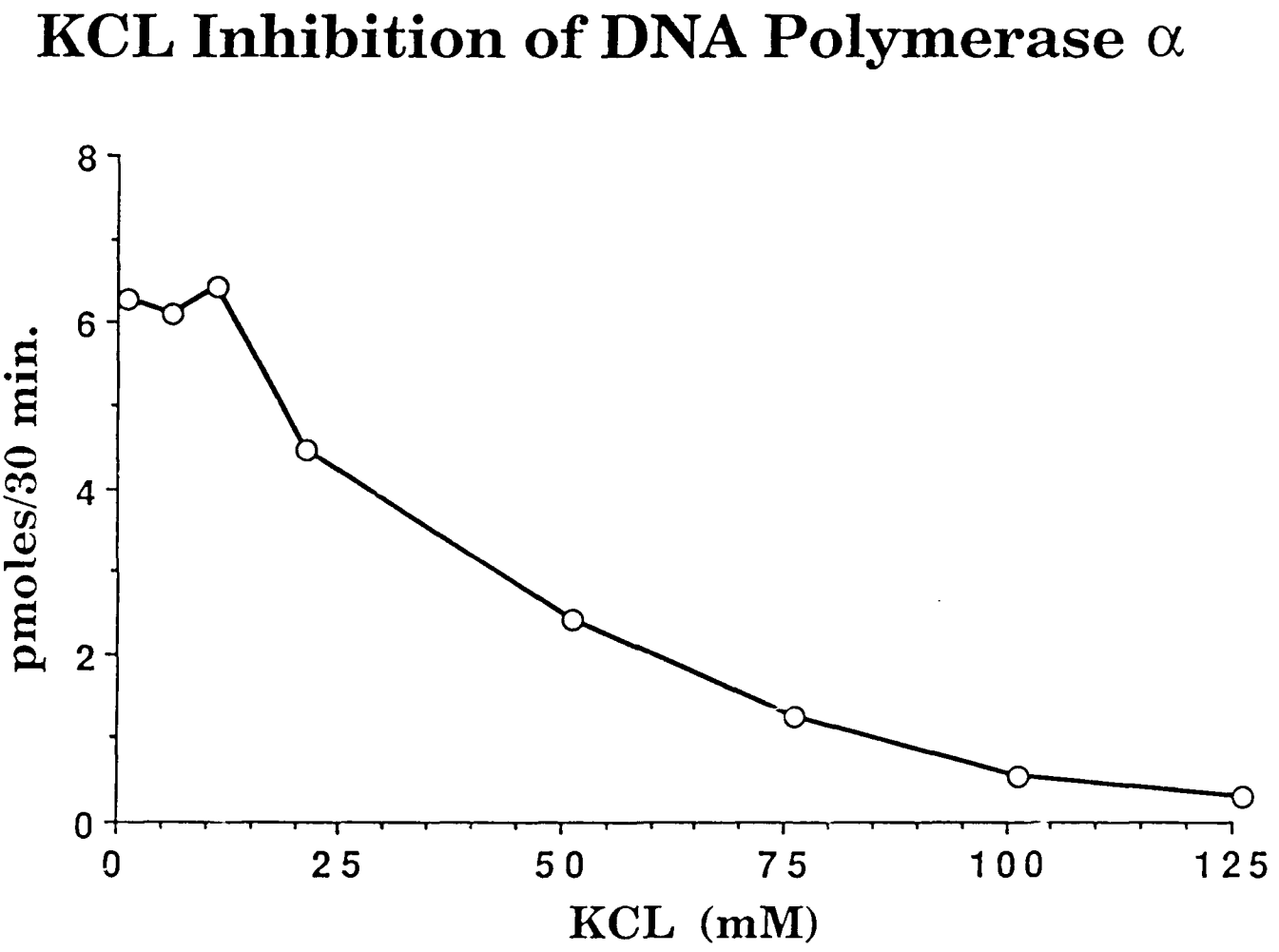

KCL Inhibition of DNA Polymerase α
pmoles/30 min.
8
6
4
2
0
0
25
50
75
100
125
KCL (mM)

Table 4

Purfication Table DNA Polymerase α

Fraction	Protein (mg)	Total Units (Units)	Specific Activity (Units/mg)	Fold Purification
Crude	630	85	0.13	1
DEAE Cellulose	130	67	0.51	4
Phosphocellulose	11.4	6,431	564	4,338
Gel Filtration	0.42	7,600	18,095	139,192

Table 5

Purfication Table DNA Polymerase β

Fraction	Protein (mg)	Total Units (Units)	Specific Activity (Units/mg)	Fold Purification
Crude	593.3	66	0.1	1
DEAE Cellulose	285.2	289	1	9
Phosphocellulose	6.4	2,040	318	2,898
Gel Filtration	0.03	22	730	6,636

Table 6

Leishmania mexicana DNA polymerase β

Compound	Ki (μM)
ddTTP	10 μm
Linoleic Acid	15 μm
Ethidium Bromide	29 μm
Berenil	115 μm
Arachidonic Acid	120 μm
BuPdGTP	160 μm
BuAdATP	200 μm
Phosphonoacetic Acid	1500 μm

- N-ethylmaleimide gave 37% Inhibition at 10 mM, the highest concentration tested.
- Aphidicolin, Phosphomycin, Sinefungin and Spermine did not inhibit the DNA polymerase β at the concentrations tested.

Figure 9

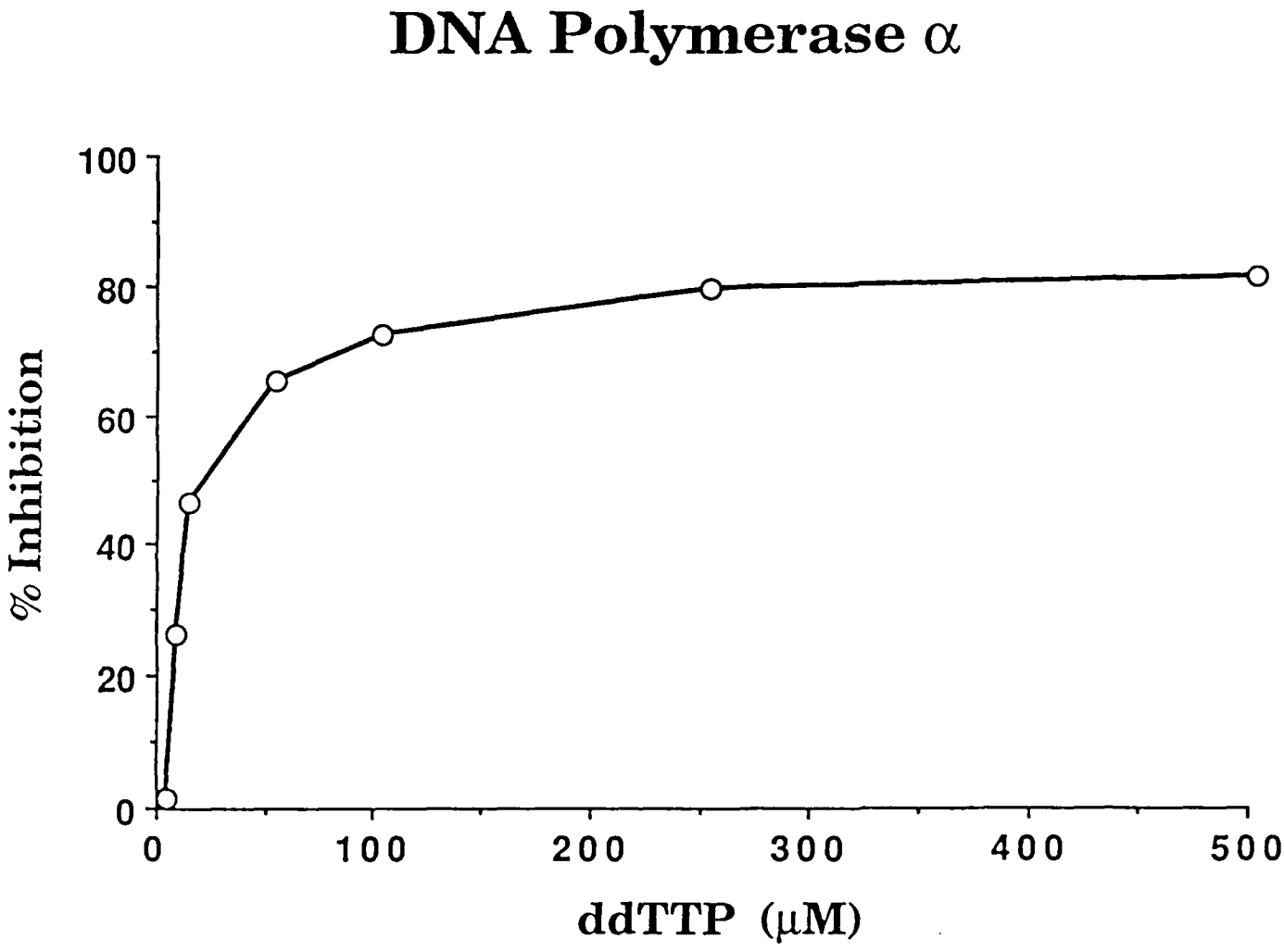

DNA Polymerase α
% Inhibition
100
80
60
40
20
0
0
100
200
300
400
500
ddTTP (μM)

Figure 10

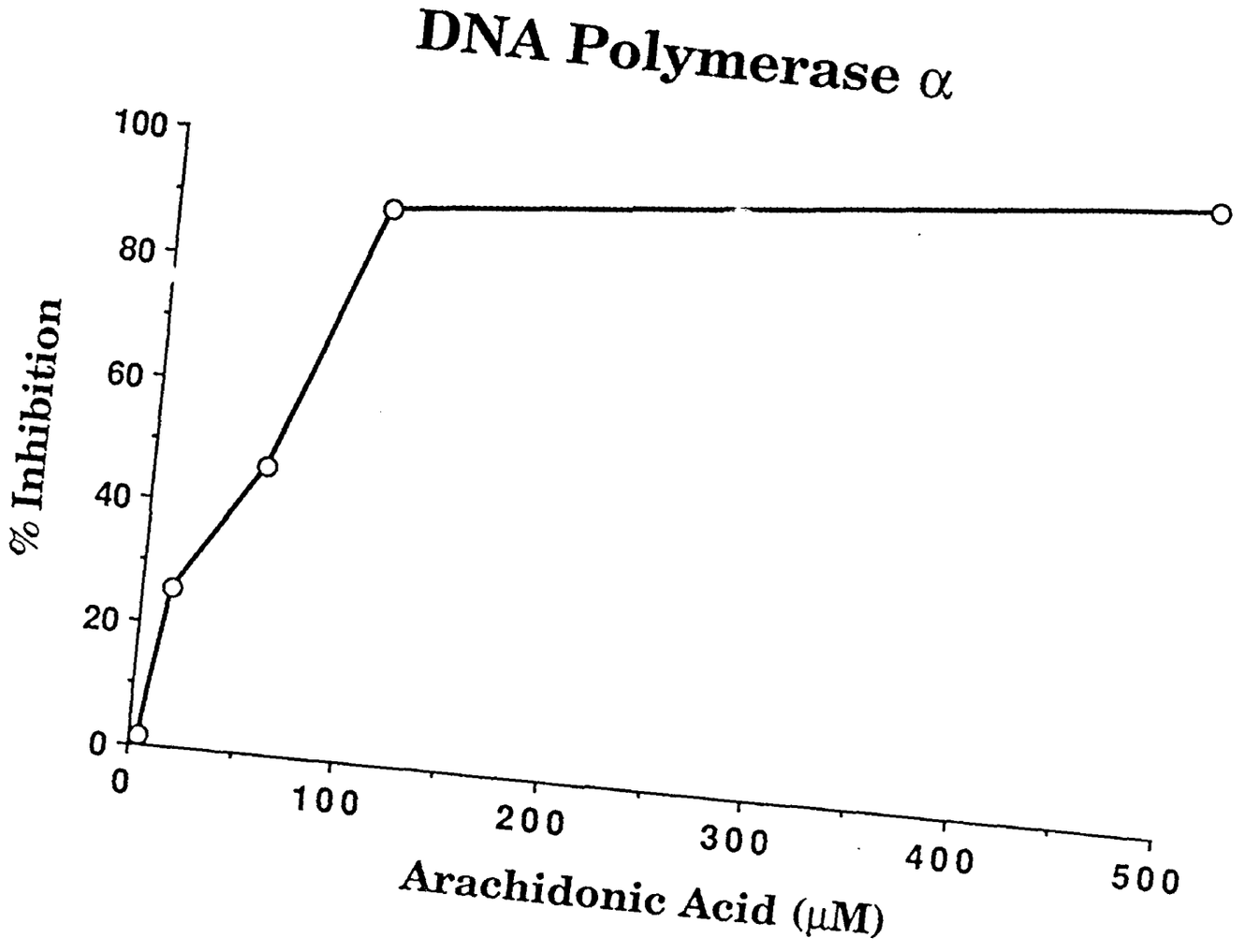

DNA Polymerase α
% Inhibition
100
80
60
40
20
0
0
100
200
300
400
500
Arachidonic Acid (μM)

Figure 11

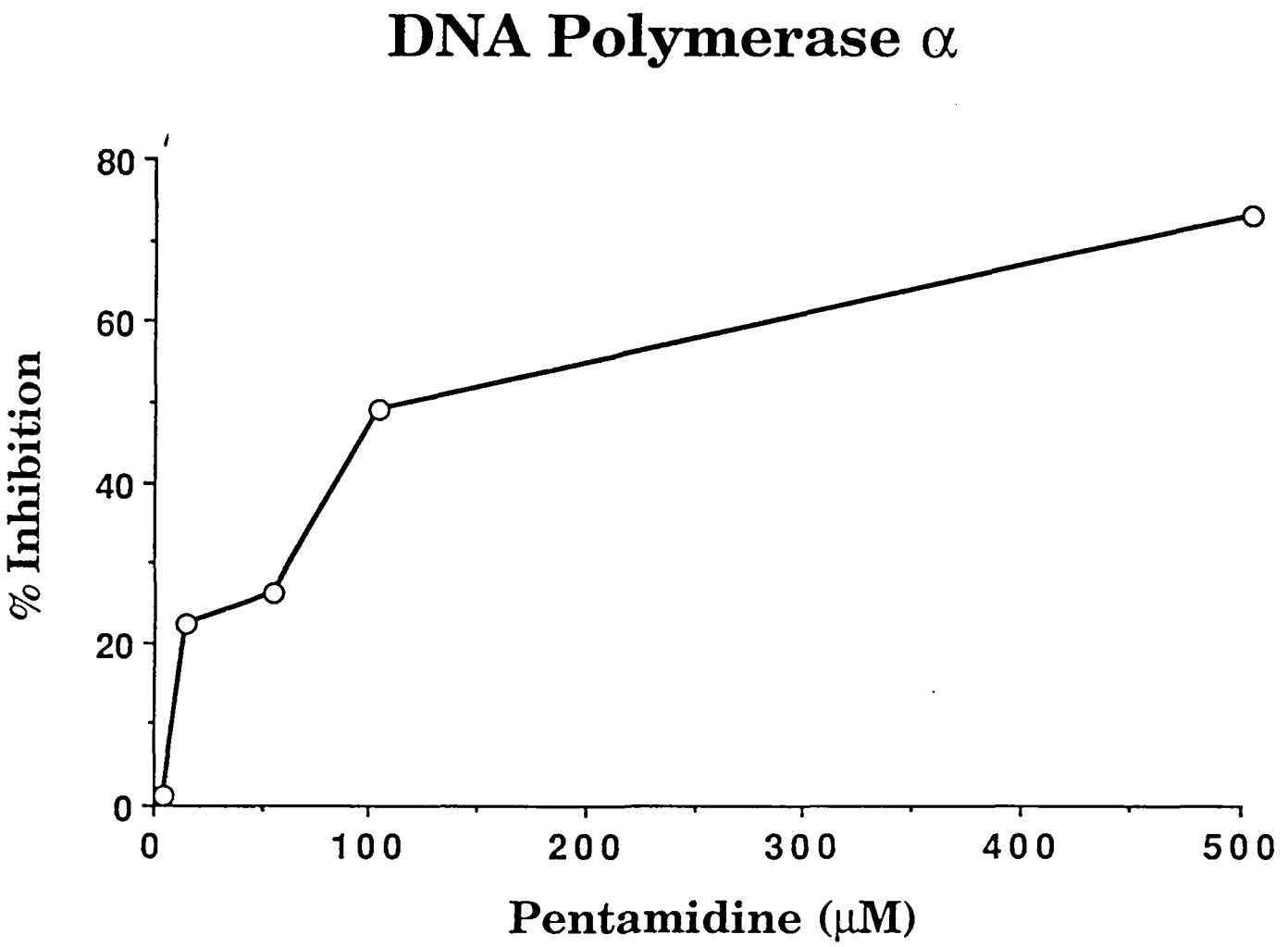

DNA Polymerase α
% Inhibition
80
60
40
20
0
0
100
200
300
400
500
Pentamidine (μM)

Figure 12

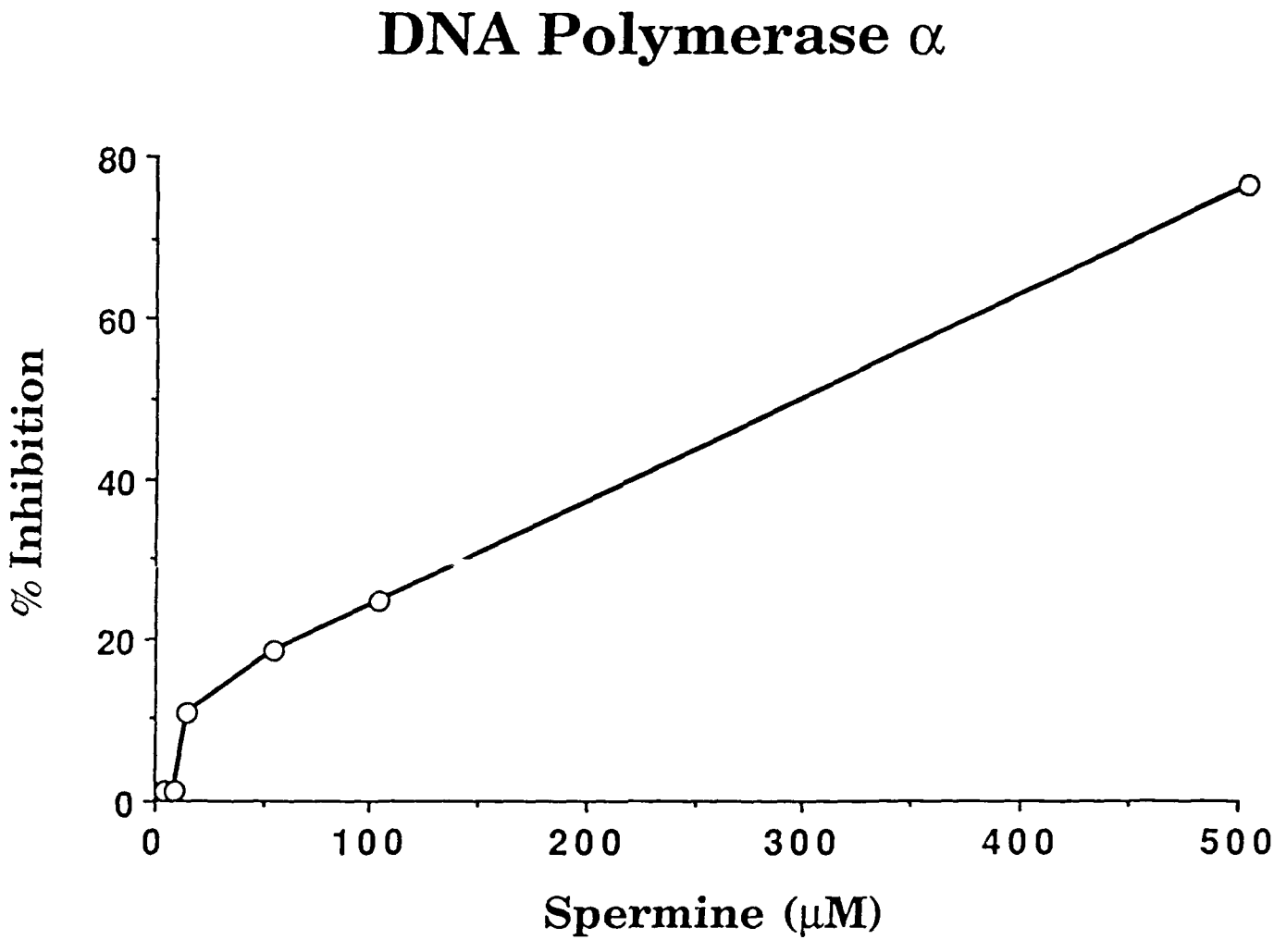

DNA Polymerase α
% Inhibition
80
60
40
20
0
0
100
200
300
400
500
Spermine (μM)

Figure 13

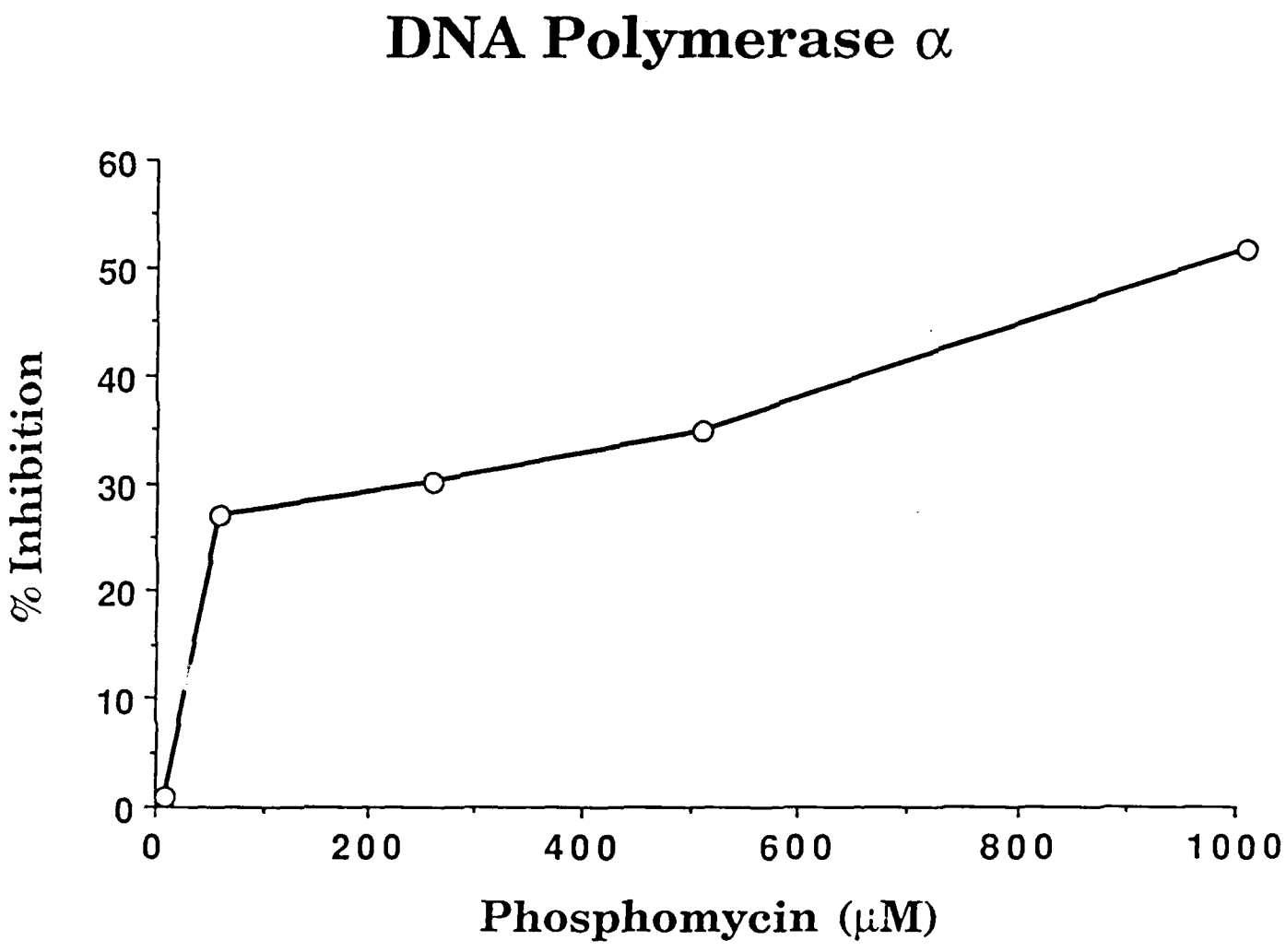

DNA Polymerase α
% Inhibition
60
50
40
30
20
10
0
0
200
400
600
800
1000
Phosphomycin (μM)

Table 7

Leishmania mexicana DNA polymerase α

Compound	Ki (μM)
Ethidium Bromide	25 μm
ddTTP	30 μm
Berenil	40 μm
Arachidonic Acid	60 μm
Pentamidine	125 μm
Linoleic Acid	160 μm
BuPdGTP	170 μm
BuAdATP	180 μm
Spermine	250 μm
Phosphomycin	1000 μm
N-ethylmaleimide	< 1250 μm

Table 8

Leishmania mexicana DNA polymerase α

Compound	Concentration	% Inhibition
Acyclovir	100 μm	0
AZT	100 μm	0
WR 24446	100 μm	0
WR 783750	100 μm	0
Ponicidin	200 μm	0
Oridion	200 μm	12
Aphidicolin	100 μm	15
Sinefungin	500 μm	21
ddI	500 μm	22
ddC	500 μm	25
ZP 65105	500 μm	33
Phosphonoacetic Acid	2000 μm	35

TABLE 9. Compounds tested for inhibition of the L. mexicana DNA polymerase α .

Name	Concentrations	%Inhibition	ID_{50}
Acyclovir	5-100μM	0	-^
Aphidicolin	5-100μM	15	ND*
Arachidonic Acid	10-500μM	25-100	60μM
AZT	5-100μM	0	-
ddC	10-500μM	25	ND
ddI	5-500μM	22	ND
ddTTP	5-500μM	25-80	30μM
Garlic Extract	2.25-45μg/ml#	18	ND
Pentamidine	10-500μM	21-72	125μM
Phosphonoacetic Acid	10 M-2mM	35	ND
Phosphomycin	50 M-1mM	51	1mM
Sinefungin	5-500μM	21	ND
Spermine	5-500μM	0-75	250μM
WR 24446	5-100μM	0	-
WR 783750	5-100μM	0	-
ZP65105	5-500μM	33	ND

^ Compounds did not inhibit the enzyme at the concentration range tested.

* Compounds that did not significantly inhibit the enzyme relative to the control over the concentration range tested were lisced as ND under the ID_{50} column.

Garlic extract is expressed in terms of protein content. Undiluted garlic extract had a protein content of 4.5 mg/mL.

Table 10

Comparison of Enzyme Sources

	BuPdGTP (ki)	BuPdATP (ki)
HeLa pol α	1 nm	<10 nm
L. mexicana pol α	170 μm	180 μm
HeLa pol β	Resistant	100 μm
L. mexicana pol β	160 μm	200 μm

- L. mexicana pol α is 105 times less sensitive to BuPdGTP and BuAdATP than HeLa pol α. Compared to L. mexicana pol β was resistant to BuPdGTP and 2 times more sensitive to BuAdATP.

Table 11

ENZYME SPECIFICITY OF BuAdATP and BuPdGTP

ENZYME	[BuPdGTP]	% Control Activity	[BuAdATP]	% Control Activity
E. Coli pol I	400 μM	100	200 μM	100
B. subtilis pol III	400 μM	100	200 μM	100
HeLa pol α	5 nM	31	5 nM	36
" " "	20 nM	8	20 nM	7
HeLa pol β	400 μM	100	5 μM	100
" " "	---	---	25 μM	86
" " "	---	---	100 μM	46
HeLa pol γ	400 μM	100	5 nM	100
" " "	---	---	25 nM	84
" " "	---	---	1 μM	66
" " "	---	---	5 μM	64
" " "	---	---	25 μM	58
" " "	---	---	200 μM	36
Rabbit Pol α	25 nM	12	25 nM	12
Rabbit Pol δ	25 nM	>90	25 nM	85

All enzymes were assayed with activated calf thymus DNA. The HeLa enzymes were assayed in standard assay with all 4 dNTPs at 100 μM each. The bacterial and rabbit bone marrow enzymes were assayed using truncated assay (dGTP-deficient where BuPdGTP was used; dATP-deficient where BuAdATP was used).

ISOLATION AND CHARACTERIZATION OF TWO ISOZYMES OF S-ADENOSYLMETHIONINE SYNTHETASE

Two isoforms of S-adenosylmethionine synthetase, α and β, have been isolated and partially purified from L.mexicana 227 cells. The crude enzymes were isolated by suspending 17g of pelletted cells in 13 ml buffer A containing 20mM potassium phosphate pH 7.5, 0.1 mMEDTA, 1mM DTT. The cells were sonicated 3x for 15 seconds, and to the cell suspension was added protease inhibitors and centrifuged at 4°C for 90 minutes at 18,000 rpm in a SS-34 rotor (39,000xg). The cell extract (20ml) was fractionated with ammonium sulfate (75% saturation). The precipitated proteins were dissolved in 17 ml of buffer A and then dialyzed at 4°C against 200 vol. of the same buffer. The dialyzed enzyme (20 ml) was applied to a DEAE-cellulose column and the above buffer was passed through the column until 280 nm-absorbing material in the eluate was negligible. The enzyme was eluted using a 160 ml linear gradient of 0-0.3M KCl in buffer A. Fractions with activity of 50% of maximum or greater were kept. The pooled DEAE-cellulose eluate (47 ml) was brought to 75% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation (39,000 xg, 30 min.) and redissolved in a minimal volume (3 ml) of buffer B containing 20 mM potassium phosphate pH 7.0, 0.2MKCl, 1 mM DTT, 0.1 mM EDTA, 20% (v/v) glycerol, 0.02% NaN_3. The enzyme suspension was concentrated and dialyzed by Centricon-10 using beffer B. It was then chromatographed on a column (1.5x67 cm) of Sephacryl S-300. As Figure 14 shows, two forms (α and β) of SAM-synthetase were eluted. The purification procedures, involving ammonium sulfate fractionation, DEAE-cellulose chromatography and Sephacryl S-300 gel filtration result in 154-fold and 46-fold purification of SAM-synthetase α and β respectively (Table 12).

The apparent molecular masses, estimated by gel filtration, are 86 KDa for α and 21 KDa for β using thyroglobulin (670 KDa), r-globulin (158 KDa), ovalbumin (44KDa), myoglobin (17 KDa) and cyanocobalamin (1.35 KDa) as marker proteins (Figure 2). The pH optima of DEAE-cellulose partially purified SAM-synthetase are 7.0 and 8.5 (Figure 15). Sephacryl S-300 isolated α and β SAM-synthetase both showed two pH optima which are same as DEAE-cellulose purified enzyme. This indicates that and enzymes are possibly cross-contaminated. We are now investigating the possibility of separating α and β enzymes by Sephacryl S-200 which gives better fractionation of protein molecules with similar molecular masses as α and β SAM-synthetase.

We are now in the process of testing anologs of sinefungin and S-adenosylmethionine.

Allicin, the major flavour product of garlic (Allium sativum L.) , is a well-known antimicrobial agent which originates from alliin (120). In Allium plants the enzyme, alliinase, and the corresponding cysteine sulfoxide, alliin, are thought to be located in different compartments. When the cell is damaged, alliinase cleaves aliin to allicin, pyruvate, and ammonium. There are several physiological processes in microorganisms which are affected by allicin such as lipid biosynthesis, RNA synthesis, or, in mammals, lowering of lipids and aggregation of platelets. Hitherto, no specific effects on some enzymes were observed, but in these cases the inhibitory potency of allicin could be overcome by sulfhydryl reagents such as cysteine or dithioerythritol. Focke (126) study on the mode of action of allicin and in search of its target enzyme investigated its effect on de novo fatty acid biosynthesis in plants. Subcellular organelles (plastids) and enzyme preparations capable of fatty acid biosynthesis from different radioactive precursors were applied. Focke (126) found the target enzyme to be acetyl-CoA synthetase.

We have found extracts of elephant garlic to be extremely toxic to Leishmania and are currently investigating the mode of action of allicin in Leishmania and hope to investigate its potential as a topical treatment to be used in combination with other chemotherapeutic treatments.

Figure 14

Elution Pattern of SAM - Synthetase α and β from Sephracryl S-300 HR

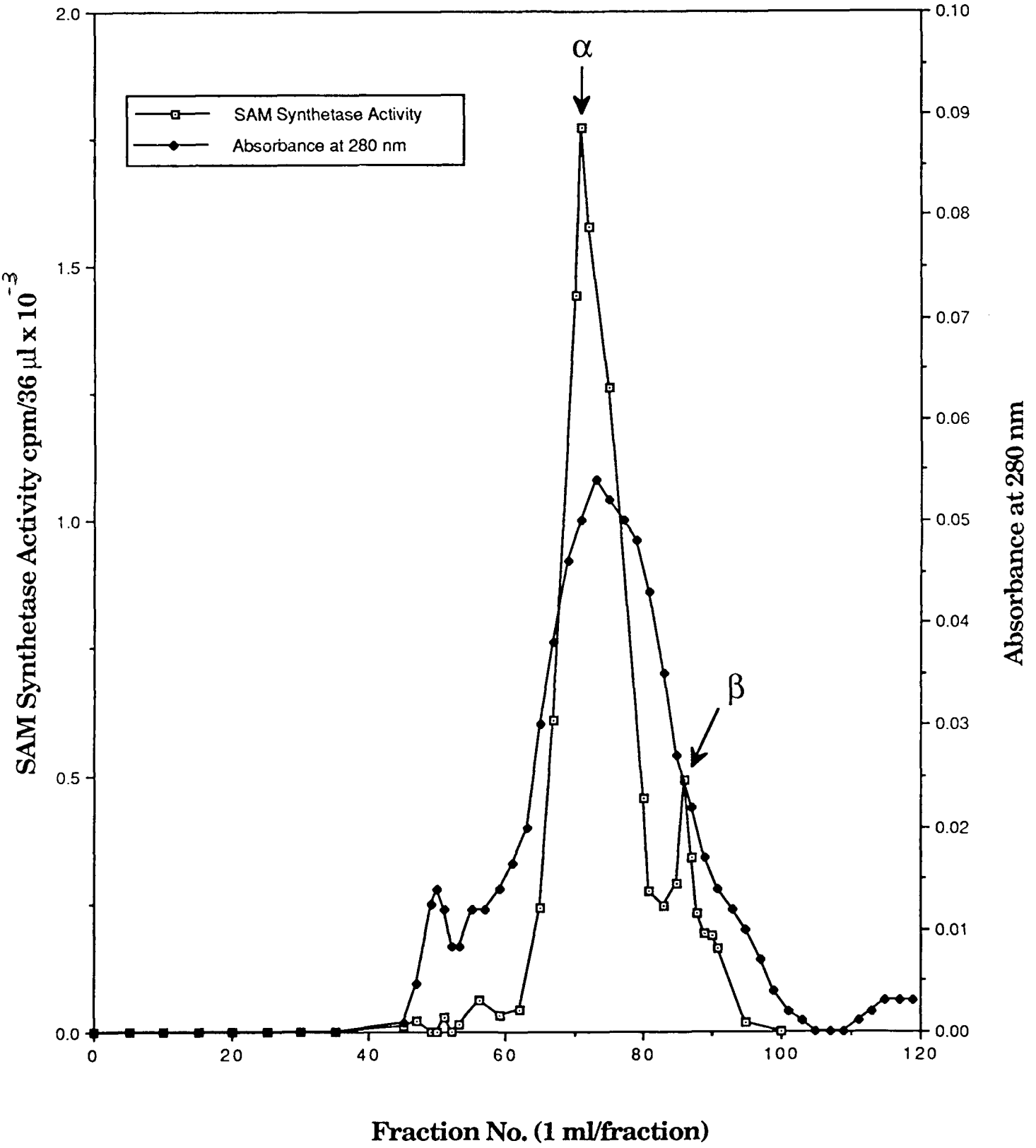
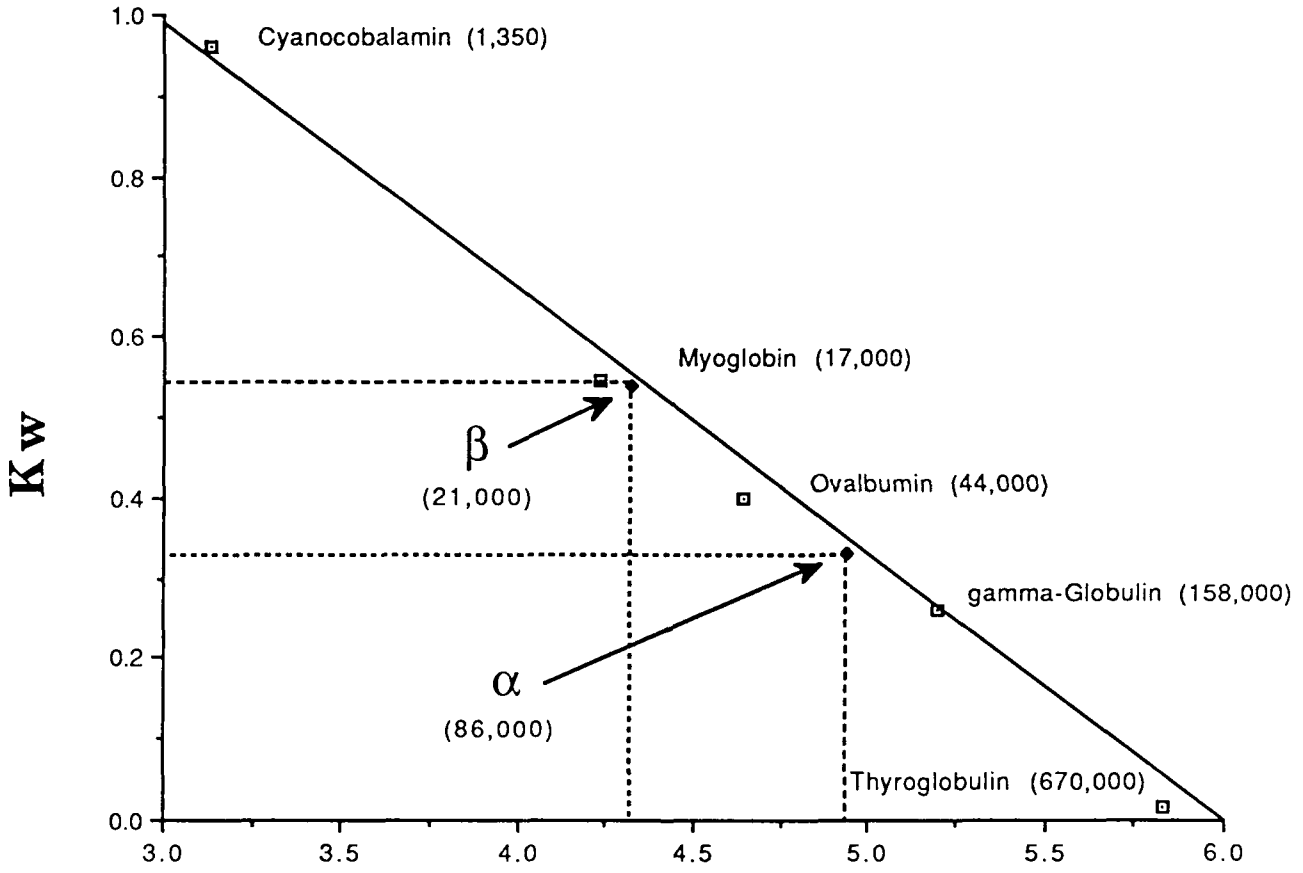


Figure 15

Molecular Weight Determination of S-Adenosylmethionine Synthetase



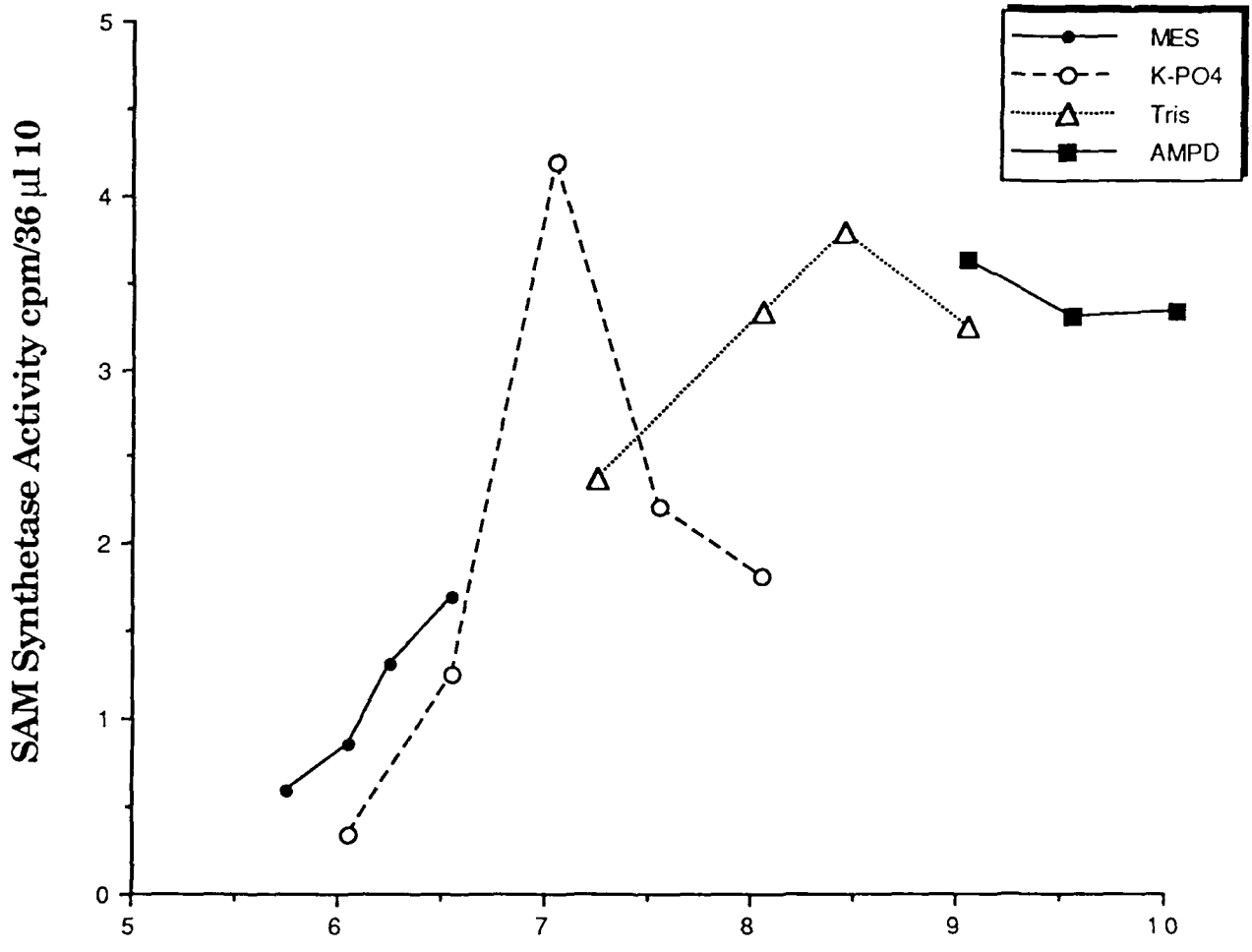
y = 2.0232 - 0.34460x R^2 = 0.996

Table 12

α & β Purification of SAM Synthetase

	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yeild %	Purification (n-fold)
Crude Extract	1024	28.8	0.028	100	1
Ammonium Sulfate Fractionation	420	176.2	0.420	611	15
DEAE - Cellulose	96	52.17	0.541	181	19.3
Sephaeryl S-300					
SAM - Synthetase α	2.45	10.58	4.32	37	154.3
SAM - Synthetase β	0.069	0.89	1.28	3	45.7

Figure 16


MES
K-PO4
Tris
AMPD
SAM Synthetase Activity cpm/36 µl 10
0
1
2
3
4
5
5
6
7
8
9
10
pH Optimum of DEAE -
Cellulose Purified SAM Synthetase

IN VIVO TESTING of POTENTIAL ANITLEISHMANIAL COMPOUNDS

I. Drug Assays in Test Tubes

A total of 34 potential chemotherapeutic drugs were tested for toxicity against Leishmania mexicana, using the test tube assay method from previous drug testing in this laboratory as described below. For comparison purposes, experimental drugs from other sources were also tested. These included azithromycin, azidothymidine, 2'3'-dideoxycytidine, dideoxyinosine, pentamidine isethionate, sulfamethoxazole, sinefungin, and in addition WR 2446, a drug sent from WRAIR which was tested previously.

Procedures and Methods.

Cultures. Promastigotes of L. mexicana amazonensis, Walter Reed strain 227 were maintained in this laboratory in tissue culture flasks containing the defined Steiger and Black medium supplemented with 5% heat-inactivated fetal calf serum and 1% gentamycin. The cells were grown at 26°C and subcultured weekly.

Assay inoculum. Cells were fed with fresh medium 24 h prior to use in assay to insure a log phase culture. The inoculum was adjusted to give an approximate starting OD of 0.1 in the assay tubes at time zero. The original inoculum should have initial readings between 0.5 - 0.7 OD at 660 nm.

Test tube assay procedure. Pyrex screw cap tubes (16X150 mm) which were scratch free and colorless were selected to match as closely as possible for use in the assay. Total volume of the test units was 5 mL. Blank tubes contained 4.5 mL medium and 500uL sterile deionized double-distilled water. Control tubes received 4.0 mL medium, 550uL water and 500 uL inoculum. Test tubes received the same as control tubes except increasing amounts of drug replaced water for a total combination of 500 uL. The standardized inoculum was stirred gently in a beaker and a 500 uL aliquot was pipetted into all but the blank tubes with an autopipette. The tubes were vortexed before reading on the Bausch & Lomb Spectronic 21 spectrophotometer at 660 nm. Tubes were incubated with loose caps in a slanted position (5°) in an incubator at 26°C for 72 hrs.

Preparation of Compounds for testing. Drugs were prepared by making a stock solution in double-distilled water. In cases where solubility was a problem, samples were made slightly acidic or basic or dissolved in 3% DMSO as needed.
Dilutions of the stock were prepare and filter sterilized.

A protein extract of elephant garlic was prepared by homogenizing a bulb of garlic in double-distilled H_2O in a Waring blender. The mixture was centrifuged at 6000 RPM and the

supernatant filter sterilized and examined for protein content. The protein concentration of the stock sample, as determined by the Bio-Rad protein assay method, was 4.5 ug /uL. Dilutions of this stock were used in the assay procedures.

Results.

The results of drug assays are presented in table form and, where ID50 was attained, in graph form. Table 13 summarizes the results of the 34 compounds sent to us from WRAIR listed in decreasing order of toxicity for L. mexicana 227. Only two of these drugs had toxic effects giving an ID_{50} or greater (Figures 17 & 18)and 10 other compounds showed evidence of some toxic inhibition. All other comounds had no activity or showed stimulation of the organism. Two drugs precipitated in the test containers giving high absorbance readings and thus incorrectly appearing to stimulate leishmanial parasites.

Table 14 lists the seven drugs tested for comparison purposes. Of these pentamidine and sinefungin were the only drugs showing strong inhibition of Leishmania. Pentamidine isethionate (Figure 19) showed inhibition at relatively low levels of drug tested, giving an ID_{50} of 42 uM. Four samples of sinefungin from three separate sources were tested and results are presented in Figure 20. These tests were performed because we noted that sinefungin samples from different sources seemed to vary in toxicity to the parasites. All four samples showed similar results at the higher concentrations (100uM or greater); however, ID_{50} values did vary. Three of the samples were very similar having ID_{50}s of 21-30 uM, while the fresly prepared Sigma sample gave an ID_{50} of 240 uM.

Elephant garlic, which has been reported to contain a powerful antimicrobial compound (allicin), was tested for toxic effects against L. mexicana. The results presented in Figure 5 show an ID_{50} of 12.5 ug protein/mL.

Conclusions.

Of the 34 WRAIR drugs tested, ZP65105 and ZP65141 show toxic inhibition which possibly warrents further testing. None of these drugs, however, were as potent as pentamidine or sinefungin against Leishmania. The samples of sinefungin should be reassayed at levels ranging from 0-50 uM, spreading out the points in the range of the ID_{50} values to get a more accurate estimation of the value.

Elephant garlic proved to be very toxic to Leishmania. We are planning to test other garlic species and correlate the results with uM of allicin present.

II. Development of an Improved Assay Procedure

An attempt was made to develope an assay method which would be technically simple and could be performed using smaller amounts of drugs. The proposed method would allow us to use 1/25 the amount of a test compound required in the test tube method. This would be a great advantage since most test compounds are in short supply.

Previously in this lab, drugs were being tested for toxicity against human CEM T4 cells using microwell plates. Results were observed by counting cells on a Coulter counter following 72 h incubation of cells in the presence of the test drug. This method had the advantage of using less test compound; however, it also was very tedious and time consuming to count cells from each well (sometimes 100 samples per assay) on the Coulter counter. Based on this fact, we attempted to assess the possibility of reading assays on a microplate reader, since optical density readings in test tube assays had proven useful and accurate as a measure of growth.

To test the theory that OD readings on the microplate reader would have a direct relationship to the number of cells in the well, several dilutions of cultures of leishmanial parasites in TRIS buffer and human CEM T_4 cells in RPMI 1640 medium were added to wells in microplates. Optical density was measured with a microplate reader and the well contents were counted on the Coulter counter.

In order to compare the new assay method with the test tube assay, the two assays were setup simultaneously to test a drug with known potency. Pentamidine was chosen as the test drug since it was very potent, easy to prepare, and we had previous results obtained in this laboratory with which to compare.

Procedures and Methods.

Assay inoculum. The inoculum was standardized at the start of the assay to an absorbance of 0.500 at 660 nm on a spectrophotometer (Bausch & Lomb Spectronic 21) in order to eliminate variations caused by different concentrations of cells growing at varying rates, and thus being inhibited to varying extents.

Microwell plate assay procedure. The assay was performed in a Corning sterile covered polystyrene 96-well round bottom tissue culture plate which was NOT tissue culture treated. This is very important because cells will stick to treated wells and the OD readings will be inaccurate. All wells contained a total of 200 uL for the assay. Blank wells contained 160 uL medium and 40uL sterile deionized double-distilled water. Control wells received 80 uL medium, 40uL water and 80 uL inoculum, while test wells received the same as control wells except increasing amounts of drug replaced water for a total combination of 40 uL. The

standardized inoculum was stirred gently in a deep Petri dish and 80 ul aliquots were pipetted into all but the blank wells with a Ranin computerized multipipette. The assay plate was shaken on a Vortex Genie-2 fitted with a 6" platform head containing a 96-well plate insert in order to insure suspension of the cells just prior to reading on the microplate reader (Dynatech, Model MR 600) previously set at # T = 410 nm. One row of control wells which had been strategically placed on the microwell plate was uncovered and suitable aliquots counted with an electronic particle counter (Coulter, Model ZF, Coulter Electronics, Hialeah, FL) for a time zero reading. The plate was then incubated in a chamber with added moisture at 26°C for 72 h and a final reading on the microplate reader and the Coulter counter was taken.

Test tube assay procedure. See previous description of procedures.

Results

Through early observations, it was quickly determined that readings in the microwell plates would need to be read at a shorter wavelength than the test tube assay. Due to the reduced absorbance of the very small volume (200 uL) in the microwells compared to the 5 mL quantity in test tubes, inhibition response would be extremely hard to detect in microwells read at 660 nm. However, as the wavelength was shortened the OD values increased. We found the response curve to have a larger slope at 410 nm than at 660 nm when measuring response in a T4 cell culture (Figure 22).

OD scans of baseline media and of cultures of T4 cells and Leishmania were observed for 72 h cultures (Figures 23 & 24). These showed that an interfering metabolite affected the curve between 350-450 nm for the T4 cell culture, but this did not occur in the Leishmania culture. This interference would have caused a decrease in the OD readings for T4 cells showing false inhibition results; therefore, it was determined that an assay for toxic effect on T4 cells should be read at a wavelenght just above this range i.e. 490 nm.

Two separate scatter plots of cultures of Leishmania and T4 cells were made using "Curve Fit" menu on Cricket Graph (Figures 24 & 25. On the scatterplots there is a direct almost linear visual relationship between the number of cells counted on the Coulter counter and the OD readings at the respective wavelengths, justifying the search for a regression. The correlation coefficient squared is indicated on Figures 24 & 25 with the correlation coefficient equal to +0.990 in both cases.

The two assay methods gave similar results for pentamidine. The ID_{50} for pentamidine as read by optical dentity compared very closely -- 40 and 48 uM (Figures 27 & 28); and these values compared favorably with our previous result of 42 uM in the test

tube assay. The Coulter readings showed a wider variation between the two methods -- ID_{50} = 22 uM in the microplate assay and was not attained at 50 uM in the test tube assay. It should be pointed out that error between samples was much greater in the microplate assay than in the test tube assay. This probably relates to the smaller volume of assay test units and the greater chance of % error in measuring volumes of the various ingredients, etc. More replicates of each sample would be advised in the microplate assay.

Conclusions.

There is strong evidence to show that the new microplate procedure would be a suitable method for use in screening potential chemotherapeutic drugs. Some of the procedures, however, need further work to increase the reliablity of results.

Initially, a baseline for test medium and for culture growth of the test organism should preceed the selection of proper wavelength for reading an assay.

In the test tube assay, the drug/water combination accounted for 10% of the total unit volume; however, in the microplate assay it was 20% of the volume. Thus, a lack of nutrients for growth of the organisms may have contributed to the toxic effect of the drugs in this assay. It is recommended that further tests of the microplate assay be tried with changes in procedures as follows:

	Microwell Assay Voulme (uL)		Test Tube Assay Volume (mL)
Ingredient	Old	New	
H_2O/Drug	40	60	0.5
Medium (1X)	80	--	4.0
Medium (1.5X)	--	80	---
Inoculum (1X)	80	60	0.5
Total	200	200	5.0
Conc. of medium in assay well as % full strength	80	90	90
Inoculum as % Total Volume	40	30	10

The use of 1.5X medium in the microwell plate assay will make the two assays more equal nutritionally and perhaps give an even better comparison of results between the two assay methods.

Table 13: Results of Drug Screening Tests of Compounds Sent from WRAIR on Leishmania mexicana

ZP #	WR #	Range Tested (uM)	% Inhibition	ID50
Strong Inhibition				
65105	240811 AA	50 - 1000	10.2 - 83.9	184 uM
65141	263527 AA	50 - 1000	33.1 - 71.8	165 uM
Weak Inhibition*				
64911	183751 AA	28.5 - 570	18.0 - 26.6	
64920	184362 AA	10 - 500	8.2 - 13.7	
65089	221656 AA	50 - 1000	12.6 - 34.3	
65150	249868 AA	33.5 - 670	10.5 - 27.1	
65169	249909 AA	33.5 - 670	8.0 - 26.5	
65178	249941 AA	50 - 1000	16.1 - 35.8	
No Inhibition				
64831	153335 AA	10 - 400		
64840	171304 AA	10 - 400		
64859	171333 AA	10 - 400		
64877	182971 AA	10 - 400		
64886	182968 AA	10 - 400		
64902	183750 AA	10 - 1000		
64957	217246 AA	10 - 500		
64975	218555 AA	7.5 - 375		
64984	218421 AA	7.5 - 375		
64993	218418 AA	10 - 500		
65007	218413 AA	10 - 500		
65025	220048 AA	10 - 500		
65034	220033 AA	10 - 500		
65114	040320 AB	50 - 1000		
65123	244633 AB	50 - 250		
65187	249940 AA	50 - 1000		
Stimulation				
64895	183119 AA	10 - 1000		
64939	184358 AA	60 - 600		
64948	185204 AA	10 - 1000		
64966	218368 AA	10 - 1000		
65016**	219984 AA	25 - 500		
65043	220001 AA	50 - 1000		
65052	221235 AA	50 - 1000		
65070	222056 AA	50 - 1000		
65098	230639 AA	50 - 1000		
65132**	249721 AA	25 - 500		
	2446	5 - 120		

* ID50 could not be obtained with concentrations tested.
** Precipitate noted in assay tubes.

Table 14: Results of Drug Screening Tests of Experimental Compounds on Leishmania mexicana

Name	Range Tested (uM)	% Inhibition	ID50
Azithromycin	5 - 1000	NE* , NE, 0.4 - 25	
Azidothymidine	50 - 900	STIM**	
Dideoxycytidine	5 - 1000	NE	
Dideoxyinosine	50 - 1000	NE	
Pentamidine isethionate	7 - 300	2.9 - 85	42 uM
Sulfamethoxazole	10 - 500	NE	
Sinefungin, Sigma 1	5 - 1000	49.2 - 69.9	30 uM
Sinefungin, Sigma 2	5 - 1000	27.6 - 59.9	240 uM
Sinefungin, Walter Reed	5 - 1000	39.9 - 66.2	21 uM
Sinefungin, Calbiochem	5 - 1000	38.0 - 63.3	24 uM
Elephant garlic (protein extract)	0.36 - 18 ug/mL	4.3 - 130	12.5 ug

* No effect
** Stimulation

Sigma 1 - Frozen sample, thawed once.
Sigma 2 - Freshly prepared sample.

Figure 17: Inhibition of Leishmania by ZP65105 (WR 240811-AA)

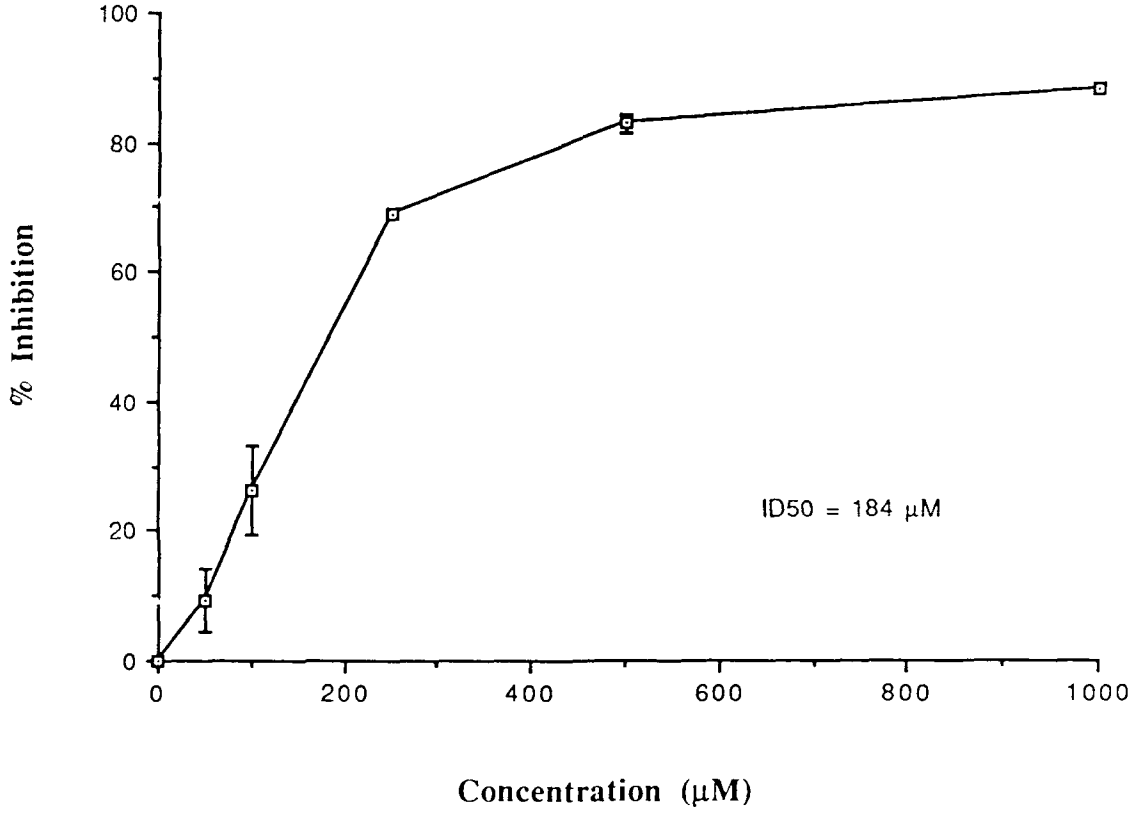


Figure 18 Inhibition of Leishmania by ZP 65141 (WR 263527-AA)

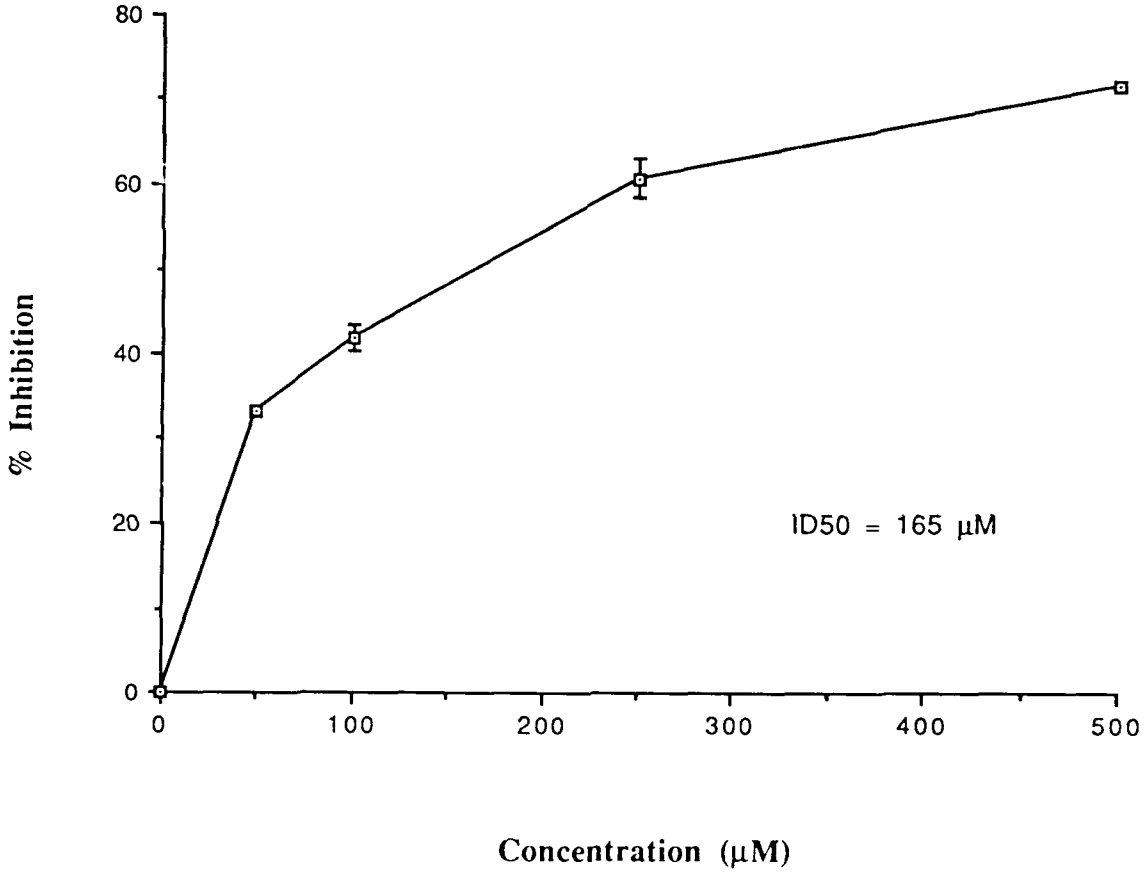


Figure 19: Inhibition of Leishmania by Pentamidine Isethionate

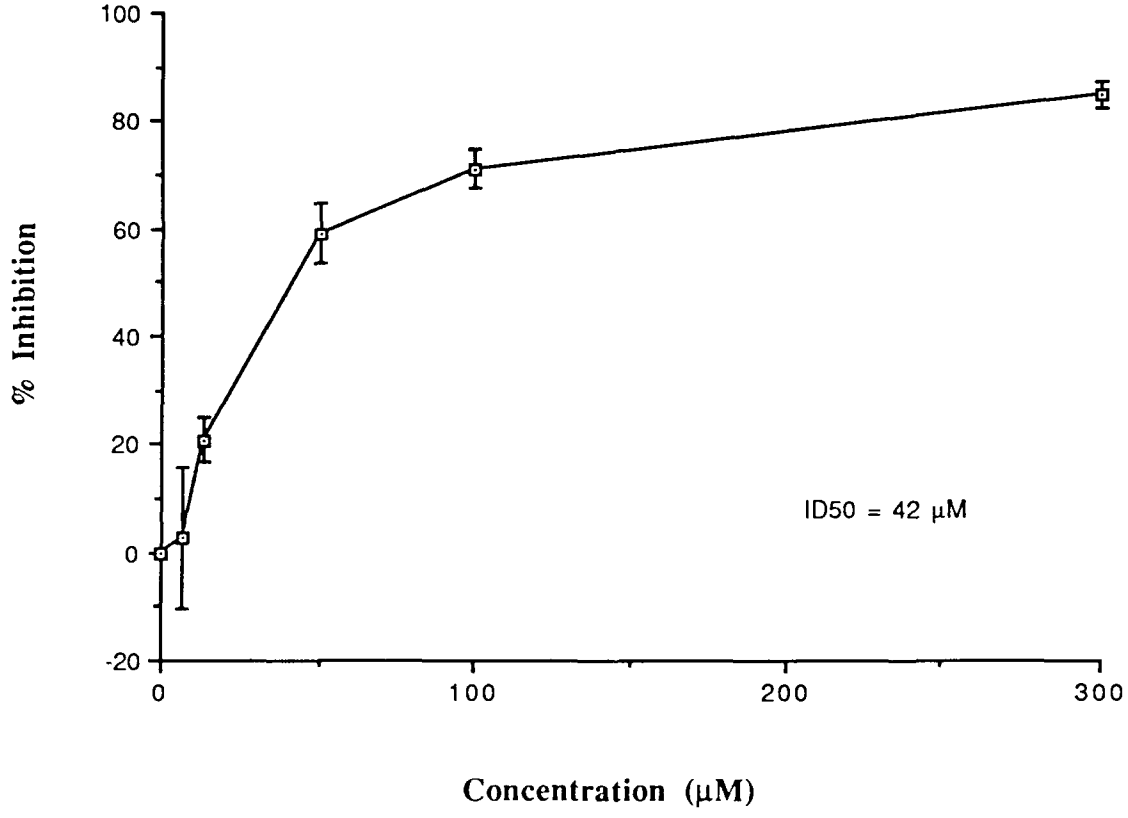
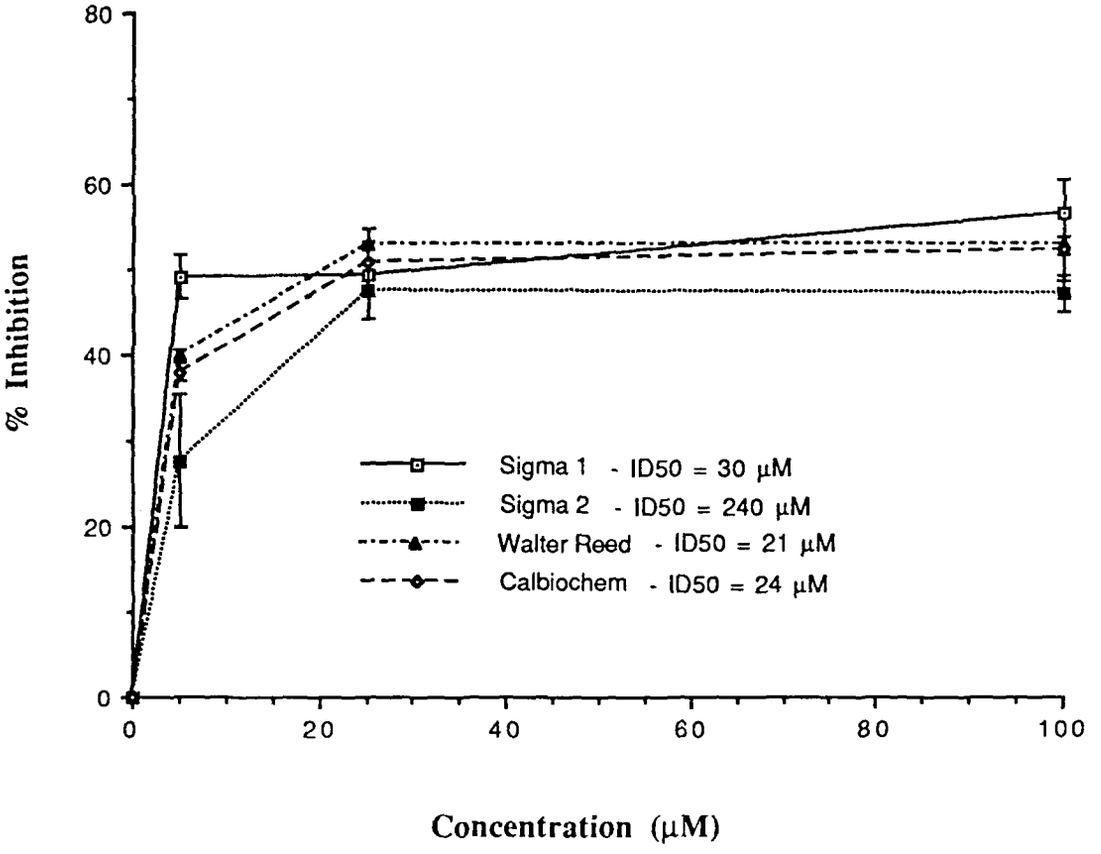


Figure 20: Inhibition of Leishmania by Sinefungin



Sigma 1 - Frozen sample thawed once

Sigma 2 - Freshly prapared from dry form

Figure 21: Toxic Inhibition of L. mexicana by an Extract of Elephant Garlic

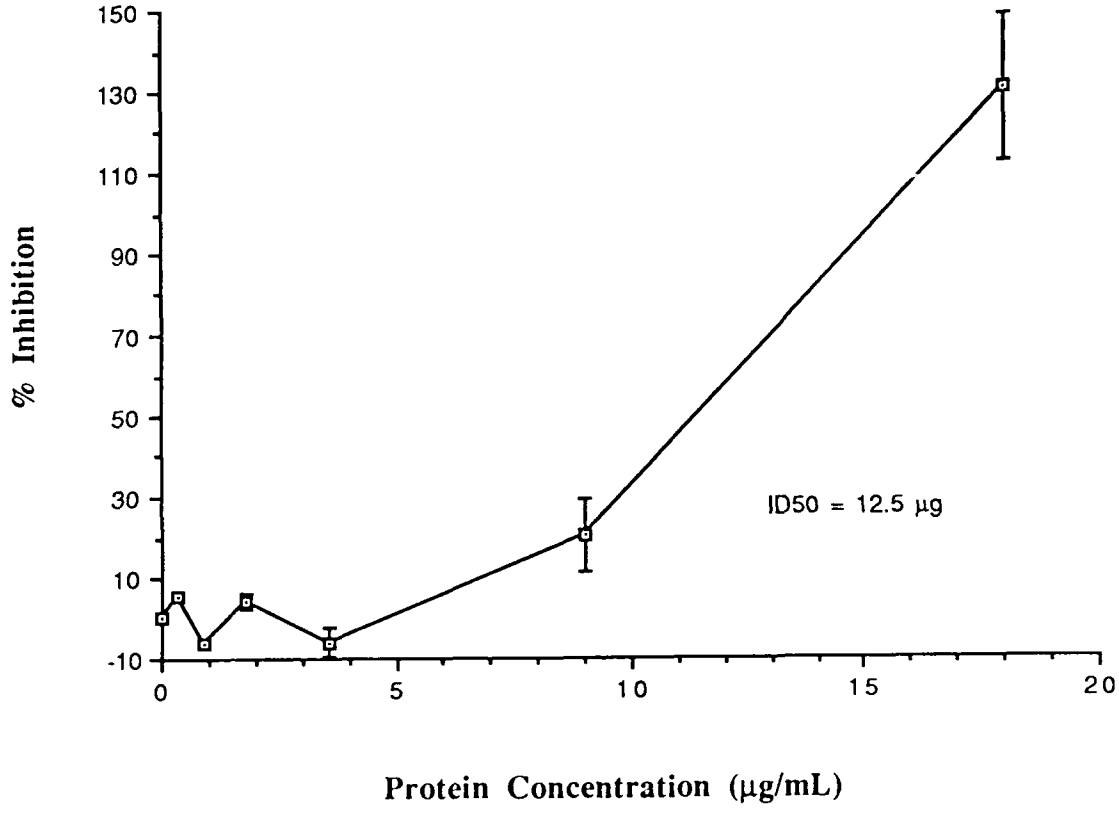


Figure 22: Optical Density Readings at Selected Wavelengths for a Culture of Human T4 Cells

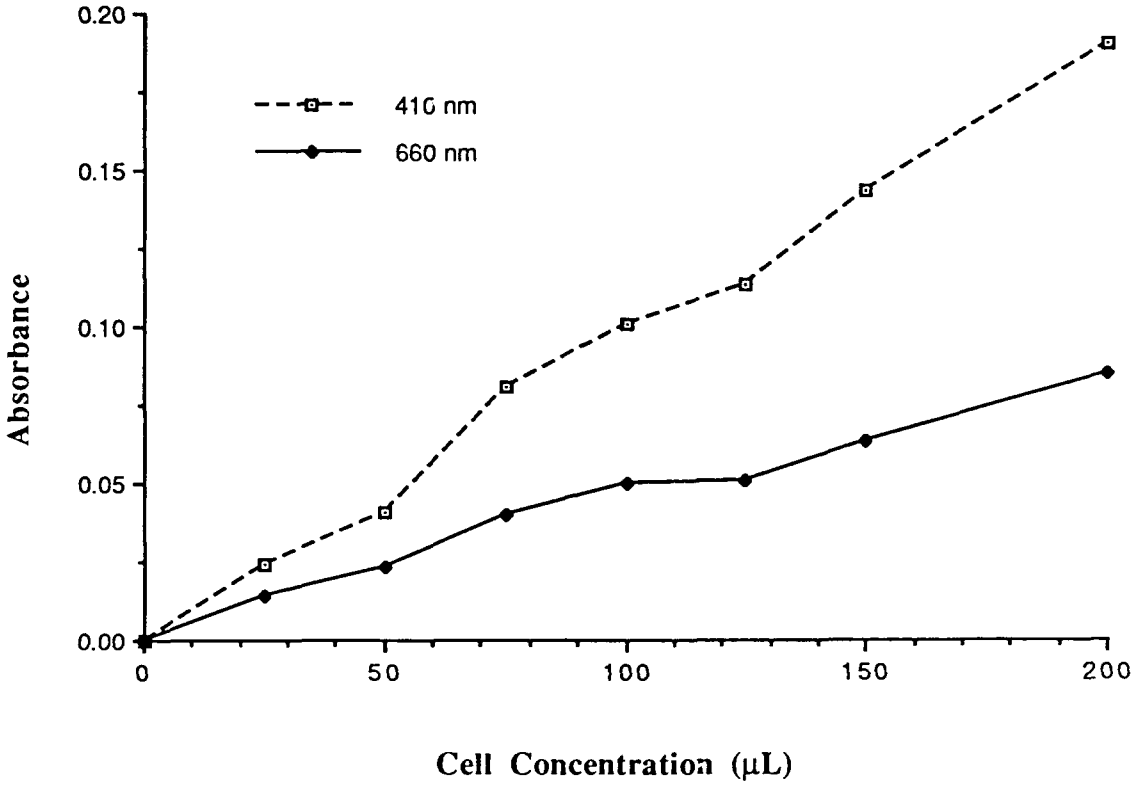


Figure 23 Optical Density Scans for Steiger & Black Medium and a 72 h Culture of Leishmania in Medium

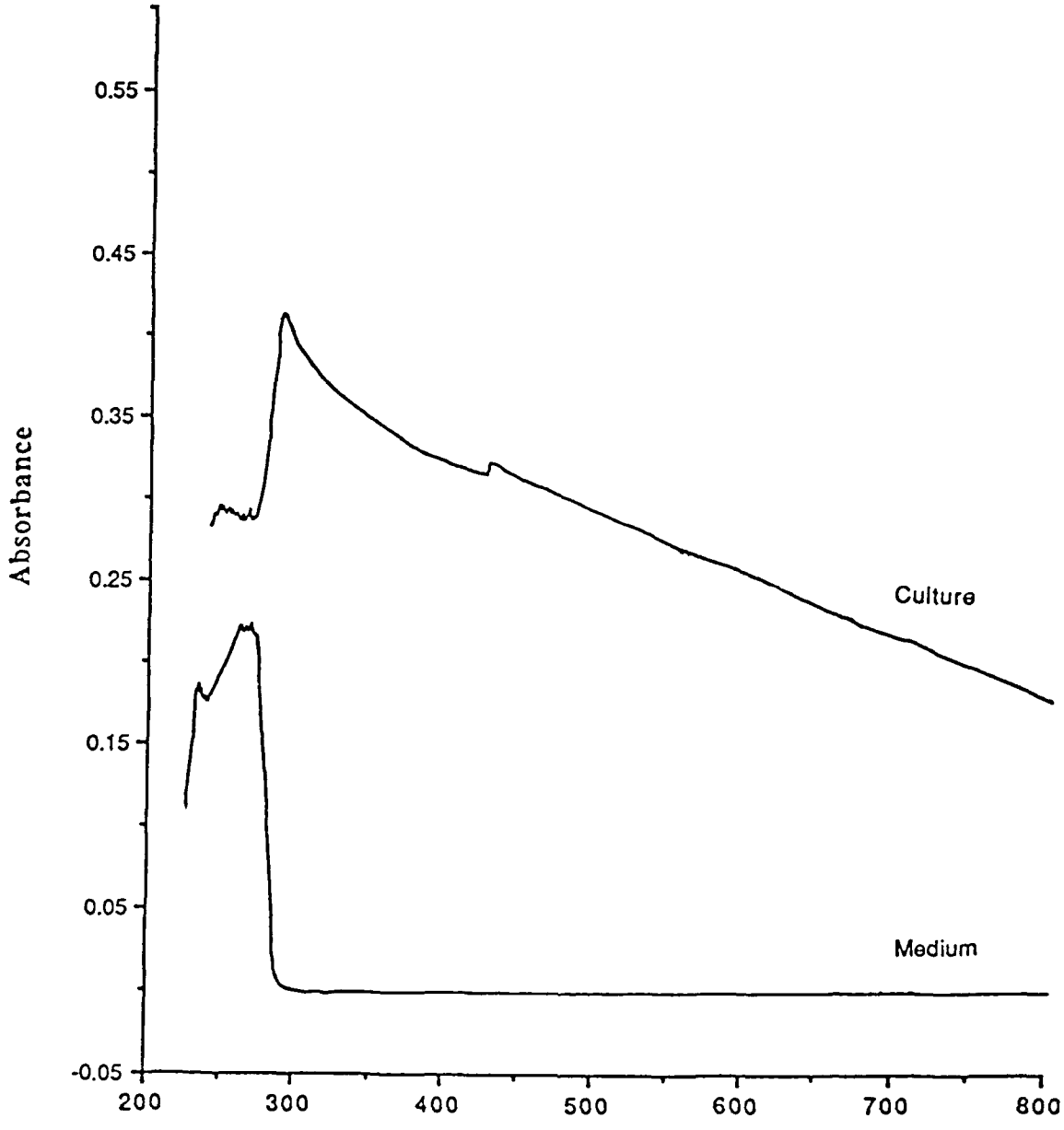


Figure 24 Optical Density Scans for RPMI-1640 Medium and a 72 h Culture of Human CEM T4 Cells in Medium

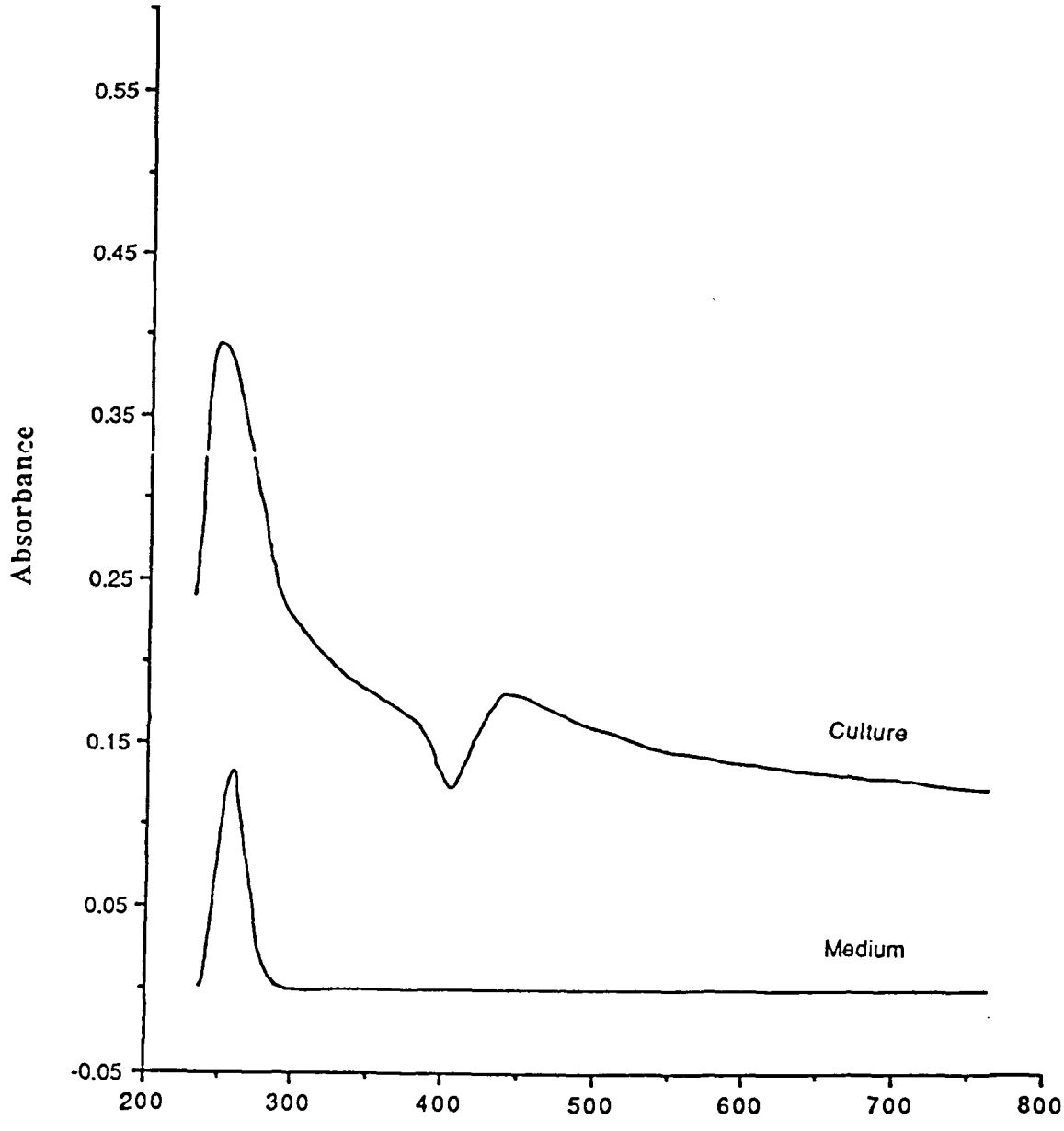


Figure 25: Relationship of Coulter Counts to Optical Density Readings of Leishmania in Buffer in Microwell Plates

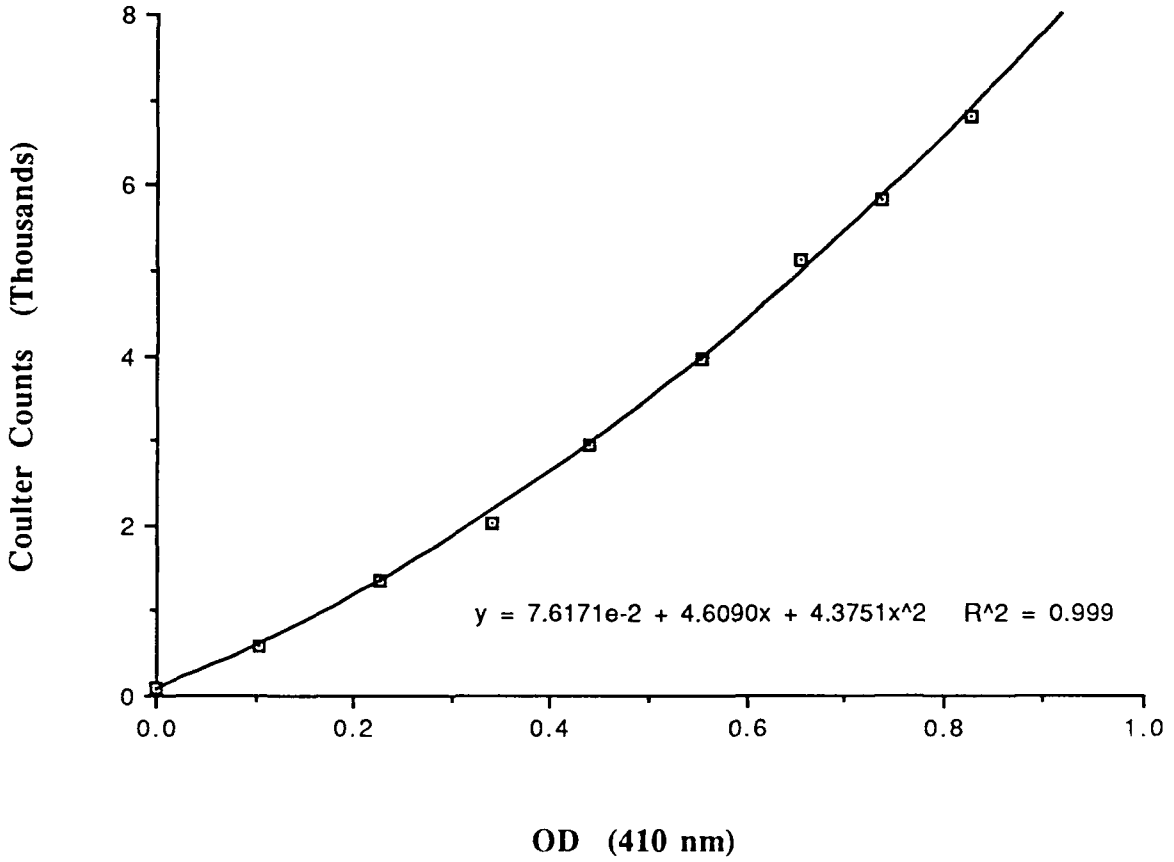


Figure 26: Relationship of Coulter Counts to Optical Density Readings for T4 Cells in Medium in Microwell Plates

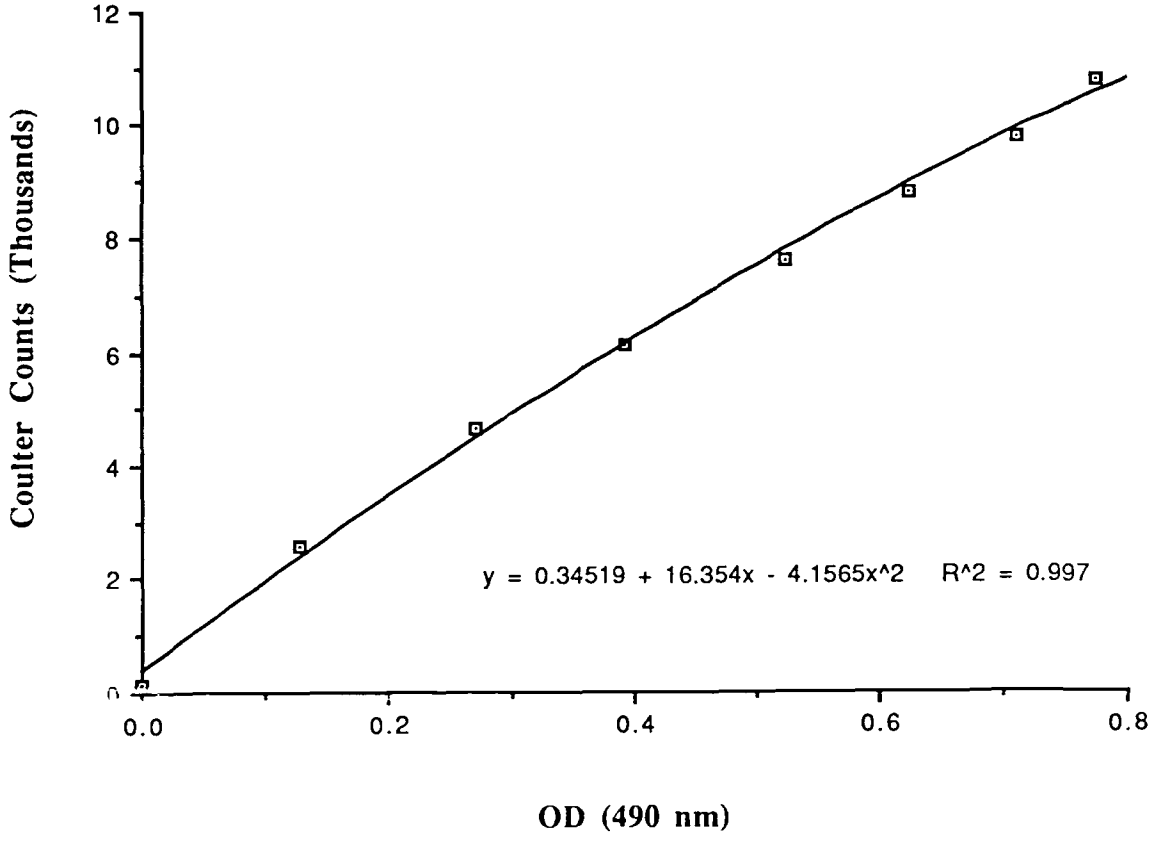


Figure 27: Toxicity of Pentamidine on L. mexicana as Measured by Test Tube Assay

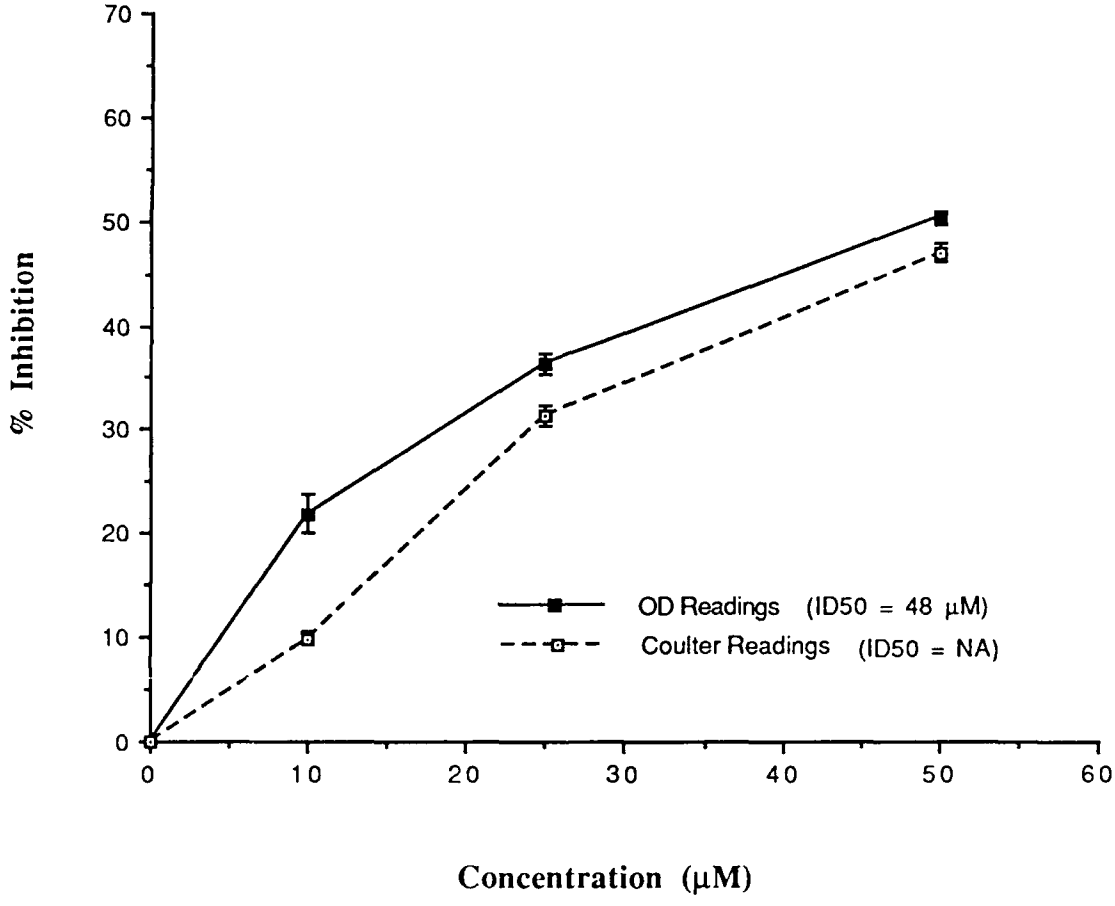
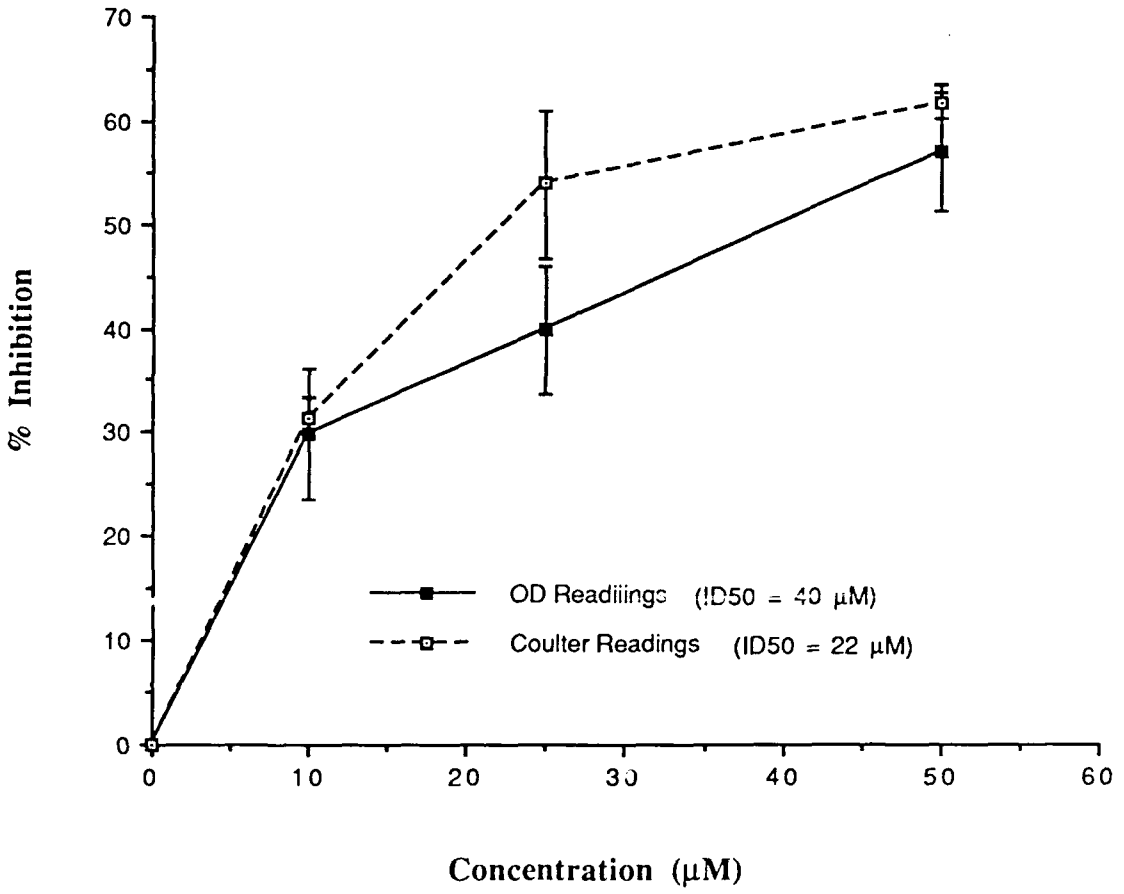


Figure 28: Toxicity of Pentamidine on L. mexicana as Measured by Microwell Plate Assay



TOXICITY OF ADENOSINE ANOLOGS SENT by WRAIR TO HUMAN T_4 Lymphocytes.

Since human T_4 cells are very sensitive to purine anologs, we choose this cell line to determine possible toxicity to potential antileishmanial compounds. The T_4 cell is essential in eliciting a proper immune response, and it would be an extreme disadvantage to compromise its function.

METHODS AND MATERIALS

The human CD4 T-cell line CEM was cultured in RPMI-1640 medium supplemented with 5% fetal calf serum, 1 gm/L $Na_{H2}CO_3$, and 50 mg/L gentamycin. Cells were assayed in 96-well polysyrene plates. The increase in cell numbers over a 72 h period was compared in wells treated with test agents relative to untreated controls. At 72 h, cultures were near the end of the logarithmic phase of growth. In each experiment, 12 control wells and 6 wells per test concentration were used. Starting cell density was standardized at 5×10^5 cells/ml by centrifugation of cells at 1,100 x g for 5 min and resuspensded in fresh medium. Toxicity to CD4 cells was measured by the concentration of an agent that causes 50 and 25 percent inhibition in CD4 cell growth relative to untreated control cells (IC_{50} and IC_{25} respectively), or alternatively by the dose of an agent that causes to and 25 percent inhibition in CD4 cell growth relative to untreated control cells (ID_{50} and ID_{25} respectively). Percent inhibition was calculated from the formula:

$$\left[1 - \frac{\text{cell growth in test wells}}{\text{cell growth in control wells}}\right] \times 100$$

Table 15, gives the toxicity data to T_4 cells exposed to test compounds sent by WRAIR.

Table 15

The Toxicity of 34 Adenosine Analogs to Human CD4 T-Lymphocytes as measured in micromoles/liter.

Compound: ZP Number	WR Number	IC_{50} (uM)	IC_{25} (uM)
ZP 64831	WR 153335 AA	>1000	390
ZP 64840	WR 171304 AA	430	253
ZP 64859	WR 171333 AA	>1000	>1000
ZP 64877	WR 182971 AA	1004	325
ZP 64886	WR 182968 AA	379	240
ZP 64895	WR 183119 AA	>1000	>1000
ZP 64902	WR 183750 AA	>1000	317
ZP 64911	WR 183751 AA	26	8
ZP 64920	WR 184362 AA	927	259
ZP 64939	WR 184358 AA	<10	<10
ZP 64948	WR 185204 AA	>1000	>1000
ZP 64957	WR 217246 AA	354	116
ZP 64966	WR 218368 AA	218	81
ZP 64975	WR 218555 AA	300	121
ZP 64984	WR 218421 AA	927	510
ZP 64993	WR 218418 AA	>1000	>1000
ZP 65007	WR 218413 AA	>1000	605
ZP 65016	WR 219984 AA	422	10
ZP 65025	WR 220048 AA	1000	44
ZP 65034	WR 220033 AA	68	16
ZP 65043	WR 220001 AA	388	10
ZP 65052	WR 221235 AA	587	58
ZP 65070	WR 222056 AA	>1000	>1000
ZP 65089	WR 221656 AA	100	10
ZP 65098	WR 230639 AA	>500	50
ZP 65105	WR 240811 AA	<10	<10
ZP 65114	WR 040320 AB	>1000	>1000
ZP 65123	WR 244633 AB	>1000	410
ZP 65132	WR 249721 AA	95	28
ZP 65141	WR 263527 AA	>1000	>1000
ZP 65150	WR 249868 AA	851	373
ZP 65169	WR 249909 AA	309	133

Table 15

ZP 65178	WR 249941 AA	546	218
ZP 65187	WR 249940 AA	>1000	529

CONCLUSIONS

(1) Higher eukaryotic cells contain at least three distinct DNA polymerases, which have been named DNA polymerases , , and . These DNA polymerases can easily be distinguished from one another by their chromatographic properties, molecular weight, sensitivity to N-ethylmaleimide and salts, and ability to copy carious templates. The structure of the eukaryotic DNA polymerases remains unresolved, although it has been shown that the - and -polymerases have molecular weights of over 100,000 and thus differ from -polymerase, which has a molecular weight of 30,000-50,000. Immunological and biochemical studies suggest that the three cellular DNA polymerases are not related to one another, do not share common peptide sequences, and are not interconvertible.

Within the past few years several laboratories have reported the existence of multiple forms of DNA polymerase from crude extracts of parasitic protozoa.

Our laboratory has purified DNA polymerase over 138,000 fold and DNA polymerase over 6,000 fold from Leishmania mexicana. The DNA polymerase were separated from on a Sephacryl S-300 column. A N-ethylmaleimide sensitive peak eluted with an approximate MW of 140,000, and a N-ethylmaleimide-resistant peak of around 40,000.

From our studies with the partial purification and characterization of DNA polymerase from Leishmania mexicana, it appears from our findings that this enzyme is very similar to the major DNA polymerase isolated from related parasitic protozoa. It is similar in characteristics to mammalian DNA polymerase regarding sensitivity to salt and N-ethylmaleimide, but strikingly different in being resistant to inhibition by aphidicolin and 2-arylaminopurine deoxyribonucleoside 5' triphosphates.

Results of our laboratory and others have demonstrated that the DNA polymerases of the related pathogenic protozoa, Trypanosomy brucei and Crithidia fasciculate are immulonogically distinct from higher eukaryotic DNA polymerase and the data suggests that structural differences between the parasite and host enzymes could be exploited for the development of agents to combat parasitic diseases.

(2) We have partially purified S-adenosylmethionine synthetase and are currently about to test anologs against this enzyme. this enzyme is necessary for the methylation of proteins, phospholipids and nucleic acids and may provide a potential chemotherapeutic target.

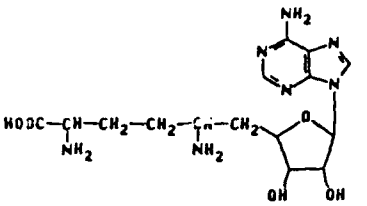
(3) Our laboratory has developed a more rapid and economical method for the screening of antileishmanial compounds against leishmanial promastigotes.

The following compounds tested during this contract year proved to be the most inhibitory in vivo.

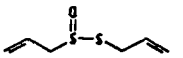
Sinefungin has been found to inhibit nucleic acid biosynthesis.

Allicin (active component in garlic extract) is thought to inhibit acetyl CoA synthetase. All other compounds were sent from WRAIR and from their structure it is most likely they inhibit nucleic acid metabolism.

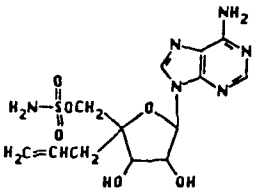
Only sinefungi, allicin, ZP65105 and ZP65141 can be considered strongly inhibitory.



Sinefungin

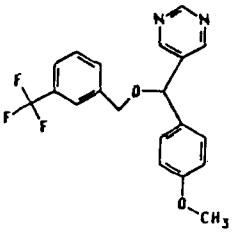


Allicin

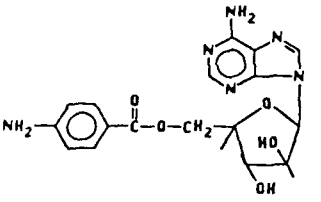


4'- (Prop-2-enyl) - 5'- O - sulfamoyladenosine

ZP65105

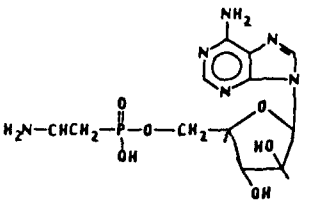


ZP65141



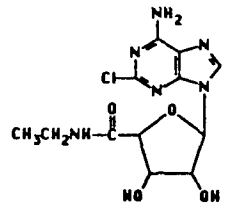
9 - (β -D-Arabinofuranosyl) Adenine, 5' - (P-Aminobenzoate) - Ester

ZP64911



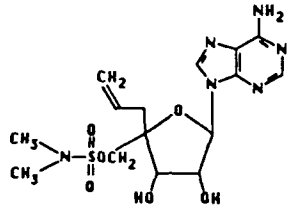
9 - β -D-Arabinofuranosyladenine, 5' - (2-aminoethyl) - phosphate, dihydrate

ZP64920



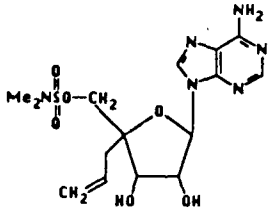
A - 44183

ZP65089



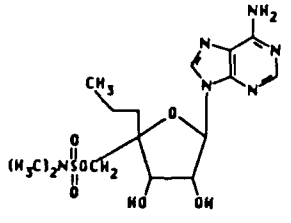
9 - [5'- O - Dimethylsulfamoyl - 4'- C - (prop - 2 - enyl) - α - L - lyxofuranosyl] - adenine

ZP65150



5'- O - (Dimethylsulfamoyl) - 4'- C - (prop - 2 - enyl) - adenosine hemihydrate

ZP65169



9 - [5'- O - Dimethylsulfamoyl - 4'- C - (n - propyl) - α - L - lyxofuranosyl] - adenine

ZP65178

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